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THE UNIVESITY OF ALBERTA

Chloroperoxidase: Continuous Production and Catalysis of Phenolic Transformations

by

Robert Duncan Carmichael

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA
FALL, 1987.

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Chloroperoxidase: Continuous Production and Catalysis of Phenolic Transformations submitted by Robert D. Carmichael in partial fulfilment of the requirements for the degree of Master of Science.

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Date: 23 September 1987.

ABSTRACT

Three strains of *Caldariomyces fumago* were immobilized as beads in 4% κ-carrageenan and tested for semi-continuous production of chloroperoxidase (CPO). Over an 80-day period, growing in defined medium, *C. fumago* strains CMI 89362 and ATCC 11925 produced enzyme concentrations of 99 and 71 mg/L respectively during six production periods of 12-14 days, while *C. fumago* DAOM 137632 produced only 24 mg CPO/L during 6 growth periods of 10 days. CPO production was unaffected by various regimens of washing between transfers.

Pigment production, fructose utilization and pH change in the immobilized cell cultures compared closely with the growth characteristics of free cell cultures. Using a 1 L airlift fermenter with continuous medium replacement of 20 mL/h (HRT=50 h), strain CMI 89362 in κ- carrageenan beads produced CPO at 40 mg/L for 11 days.

By keeping the inoculum size between 0.5-1.0 g wet weight/L, strain CMI 89362 could be induced to form mycelial pellets without the physical support of the κ-carrageenan. In mycelial pellet form, strain CMI 89362 also produced CPO in a airlift fermenter and was subjected to various flow rates and different media in continuously-fed airlift fermenter culture. Optimum CPO production rates, the average being 1.19±0.09 mg CPO/h, occurred at flow rates between 8.5 and 65 mL/h. The composition of the feed solution, either 2% or 4% fructose-salts, did not significantly alter the amount of CPO produced. Batch fermentation in the tower fermenter resulted in the highest CPO levels (400-500 mg/L), double the amount obtained by free hyphae in shake flask culture.

Purified CPO was used to oxidize a variety of phenolic compounds at pH 2.75 and 5.5. Under conditions of excess enzyme, chlorophenols were oxidized most readily, followed by 4-ethylphenol and then the dimethylphenols. Cyclohexanol and its monomethyl derivatives were not oxidized. Further characterization and optimization studies are required to test the feasibility of CPO for use in the enzymic treatment of industrial wastewaters.

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The suggestions and advice given by Dr. M.A. Pickard were happily received and crucial to the completion of this project. For many experiments, technical assistance provided by Atsumi Hashimoto was timely and indispensable. It was a pleasure to work with both of the above over the many years I was here.

I gratefully acknowledge all the committee members for the time and thought spent on the constructive criticism of this thesis.

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LIST OF ABBREVIATIONS

Abbreviation	
ATCC	American Type Culture Collection
BBL	registered trademark of BBL Microbiology Systems, Becton Dickinson and Co.
BRL	Bethesda Research Laboratories
CHO	carbohydrate
CMI	Commonwealth Mycological Institute
CPO	chloroperoxidase
DMSO	dimethylsulfoxide
DOAM	Mycological Herbarium Division of Botany and Plant Pathology, Department of Agriculture, Ottawa, Canada.
cDNA	complementary deoxyribonucleic acid
ELISA	enzyme-linked immunosorption assay
EU	enzyme unit(s)
FL	fungal laccase
FS	fructose salts medium
GC	gas chromatography
HRP	horseradish peroxidase
HRT	hydraulic retention time (system volume/flow rate)
IEF	isoelectric focusing
LPO	lactoperoxidase
PAGE	polyacrylamide gel electrophoresis
PDA	potato dextrose agar
PEG	polyethylene glycol
PFS	phytone fructose salts medium

Abbreviation	
PID	proportional, integral, and derivative
PPO	polyphenol oxidase
mRNA	messenger ribonucleic acid
Rz	Reinheitszahl (A_{403} nm/A_{280} nm)
UAMH	University of Alberta Mould Herbarium

1. INTRODUCTION AND LITERATURE REVIEW

Fungal fermentations for enzyme production are now being widely studied as shown by the large number of papers published in this area, especially on the production of amylases, cellulases, ligninases and proteases. However chloroperoxidase (CPO), and the haloperoxidases as a whole group, which have been well studied biochemically, have not received as much attention in the literature from the production viewpoint. This perhaps has been due to the lack of knowledge of the genetics and physiology of major enzyme producing fungi (Priest 1983). Most industrial fungal fermentations and studies are carried out on an empirical level where the amount of product formed is the major and often only concern.

When this project began, it was known that extracellular enzymes could be produced continuously and repeatedly by immobilized cells (Kobuku *et al* 1978; Kobuku *et al* 1981; Frein *et al* 1982) and that haloperoxidases and related enzymes were capable of catalyzing the oxidation of carcinogenic aromatic amines (Klibanov and Morris 1981) and phenolic compound compounds (Klibanov *et al* 1983) which led to the proposed use of enzymes for the treatment of phenolic wastewaters (Alberti and Klibanov 1981). In particular, CPO has been available for a long time from commercial sources and was known to be able to catalyze the oxidation of *p*-cresol and more recently benzidine (Alberti and Klibanov 1981). However no published data existed for the large scale production of CPO in fermenters, the method of choice used by industry, and the extent of the ability of CPO to oxidize phenolic compound compounds had not been elucidated.

Initially this project was an attempt to develop techniques to provide regularly a low cost fungal metabolite for collaborative physiochemical studies on enzyme reaction mechanisms. In a mycology laboratory, it quickly becomes apparent that the maintenance and propagation of fungi often require different techniques than those used for bacterial culture. Many methods of fungal cultivation are available including solid-phase, stationary, shake flask, and fermenter liquid culture, each having their own specific requirements for inoculum preparation. Evidently the lack of a unified approach to the reproducible cultivation of fungi has perpetuated the use of the empirically-based study and dissuaded workers from entering the field. A prime example of a study which is empirically-based would be the penicillin fermentation. This project therefore attempts to develop a method for the effective scale-up of fungal culture for CPO production and goes on to determine if immobilized cells, showing good enzyme production levels, could be used repeatedly in shake flasks and fermenters to produce CPO.

The intent of this review is to outline first the properties of haloperoxidases generally and CPO in particular, microbial halogenation, and a chronological review of the papers by selected workers in the area. This will be followed by a survey of the literature related to this project on the topics of fungal growth, phenol oxidation and enzyme immobilization. It will end with a discussion of the rationale and purpose of the project.

For more information on specific areas please refer to the following listing of relevant publications. The literature on biohalogenations has been reviewed (Petty 1961; Neidleman, 1975; Morrison and Schonbaum, 1976; Neidleman and Geigert 1983; Neidleman and Geigert 1986). The book by Neidleman and Geigert (1986) contains comprehensive information from 411 papers on the properties, reactions and reaction mechanisms, roles and potential applications of haloperoxidases. Thus only those details directly related to this project will be discussed and subsequent publications will be noted.

Methods for cell immobilization (Tosa *et al* 1979; Cheetham 1980; Chibata and Tosa 1981) and enzyme immobilization (Falb 1972; Messing 1975) and their potential use (Linko and Linko 1984; Scott 1987) and practicality in industry (Bucke 1983; Klibanov 1983) also have been well documented and therefore will only be briefly discussed. Many topics of industrial microbiology were well covered by a recent issue of Enzyme and Microbial Technology (January, 1987) and the September, 1981 issue of Scientific American.

1.1 Haloperoxidases and Chloroperoxidase

Haloperoxidases are widespread in nature (Yamada *et al* 1985a), relatively stable (Pickard unpublished data; Wever *et al* 1985) heme proteins that are able to catalyze the oxidation of halide ions (not F^-) and a wide variety of reactions (Yamada *et al* 1985; Geigert *et al* 1986) with the formation of many unique molecules (Geigert and Neidleman 1985). Some of reactions catalyzed by CPO are shown in Fig. 1. These enzymes have been found to lack some of the general properties of enzymes such as substrate specificity, reaction reversibility and stereospecific product formation (Neidleman and Geigert 1986).

Specifically, chloroperoxidase contains one ferriprotoporphyrin IX per molecule and has a molecular weight of about 42,000. It is an extracellular glycoprotein with a variable carbohydrate content of 13-17% arabinose and glucosamine (Morris and Hager 1966) resulting in 3-6 isoenzymatic forms (Hashimoto and Pickard 1984). Amino acid analyses have shown the protein is like other haloperoxidases in its predominance of acidic over basic amino acids (Morris and Hager 1966; Neidleman and Geigert 1986). In halogenation reactions, CPO is most active as a chlorinating enzyme with a pH optimum near 3 but it can also oxidize iodide and bromide ions. In addition it can carry out a peroxidase reaction in the absence of halide substrates and unlike all other haloperoxidases, it has a catalase activity (Thomas *et al* 1970a) in addition to a halide dependent pseudo-catalase activity (see Fig. 2). Most substrates of CPO are characterized by the presence of an activated hydrogen, which is either α to a carbonyl or phenolic compound hydroxyl or on a sulfur atom (Ashley and Griffin 1981).

The discovery that haloperoxidases are able to oxidize phenols and halogenate alkenes has resulted in the proposed use of these and related enzymes in wastewater treatment (Alberti and Klibanov1981; this thesis), specialty chemical synthesis (Geigert *et al* 1983a), as pharmaceutical agents, and for analytical diagnostics (Neidleman and Geigert 1986). CPO may have potential applications in wastewater treatment, diagnostic ELISA techniques and in the commercial production of α,β-halohydrins, epoxides, steroids, and chlorinated barbituates (Neidleman and Geigert 1986; Laane *et al* 1986). Also, Martyn *et al* (1981) have studied the ability of CPO to chlorinate a variety of beta-diketones forming antimicrobial compounds. The exploitablity of enzymes and in particular haloperoxidases has not been overlooked, since patents have been filed on many reactions catalyzed by these enzymes (Neidleman and Geigert 1981; Neidleman *et al* 1981; Orndorff 1983; Geigert and Neidleman 1986). The implementation of these potential enzyme catalyzed reactions on an industrial scale would require the production of gram to kilogram quantities of enzyme.

1.2 Microbial Halogenation

Compounds containing halogens are widespread in occurrence and show remarkable chemical diversity. The number of documented halometabolites from microbial, fungal and marine origin has grown from 1 to over 700 in the last 100 years. Many of these naturally occurring halogenated substances are bio-toxic.

The large number of halogenated compounds implies the presence of a group of enzymes which are just as broad ranging and diverse. However this is not the case. Although haloperoxidases are widespread in nature, each type of haloperoxidase seems to come from a particular group of organisms. For instance, chloroperoxidases are found in dematiaceous hyphomycetes, bromoperoxidases from red algae, iodoperoxidases from brown algae. (Neidleman and Geigert 1986). Furthermore, there seems to be only one dominant type of halogenating enzyme responsible for the multitude of halometabolites.

There are over 60 natural sources of haloperoxidases (Geigert and Neidleman 1985) and the number is increasing. It was only a few years ago that sources of chloroperoxidase besides *Caldariomyces fumago* were described such as *Cryptosporiopsis* (Neidleman and Geigert 1986). Prior to that Hashimoto and Pickard (1984) had looked at 26 fungal cultures, ten being *Caldariomyces* (=*Leptoxyphium*), and found only the *Caldariomyces* cultures produced CPO.

Figure 1. Various reactions catalyzed by CPO.

1) CPO-catalyzed chlorination of monochlorodimedone to dichlorodimedone. This reaction can be followed spectrophotometrically at A_{275} nm. It was used throughout this thesis to determine enzyme activity in culture supernatants (Hager *et al* 1966). The reaction was performed using 3 ng of CPO in a 1 mL reaction mixture.

2) Reaction of CPO with ethylene to form an α,β-halohydrin (Geigert *et al* 1983a).

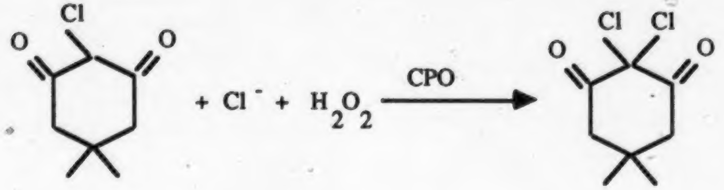
3) Schematic flow diagram of the catalase activity of CPO (Neidleman and Geigert 1986). CPO and other peroxidases are also capable of a halide-dependent catalase-like activity (see Fig. 2)

4) Schematic flow diagram of the peroxidase activty of CPO (Neidleman and Geigert 1986). The formation of radical (*) intermediates can lead to couplings and polymerization. AH can be many reducing substrates.

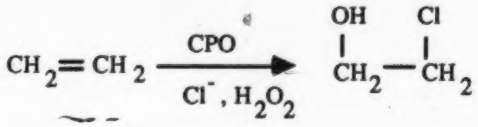
5) An example of halide-independent peroxidation of aromatic compounds (Neidleman and Geigert 1986). Oxidation of *p*-cresol to a dimer.

6) Halogenation of aromatic compounds by CPO (Geigert and Neidleman 1985). Formation of chlorinated products from anisole.

1)

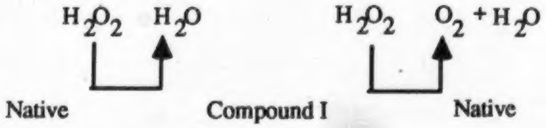

Cl
O
O
+ Cl⁻ + H2O2
CPO
Cl
Cl
O
O

2)



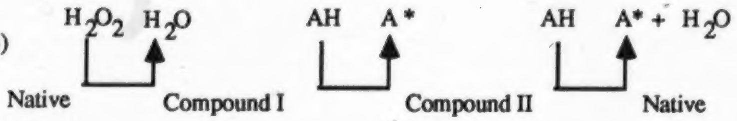
$$CH_2{=}CH_2 \xrightarrow[Cl^-,\ H_2O_2]{CPO} \overset{OH}{CH_2}-\overset{Cl}{CH_2}$$

3)


H2O2
H2O
Native
Compound I
H2O2
O2 + H2O
Native

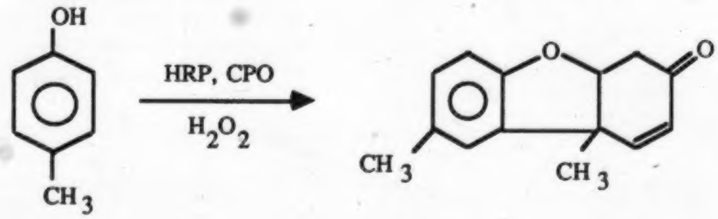
overall = $2H_2O_2 \xrightarrow{CPO} O_2 + H_2O$

4)

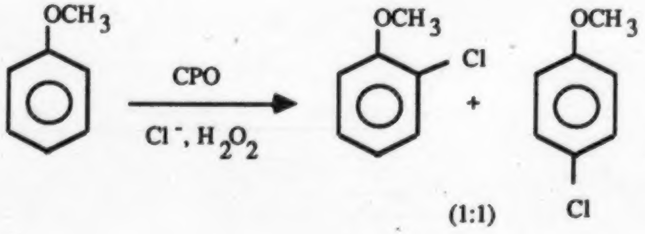

H2O2
H2O
Native
Compound I
AH
A*
Compound II
AH
A* + H2O
Native

overall = $H_2O_2 + 2AH \xrightarrow{CPO} A_2 + 2H_2O$

5)


OH
CH3
HRP, CPO
H2O2
O
O
CH3
CH3

6)


OCH3
CPO
Cl⁻, H2O2
OCH3
Cl
+
OCH3
Cl
(1:1)

Pseudomonas pyrrocinia although this enzyme differs from the fungal enzyme in its chlorinating activity (Wiesner *et al* 1986). Bromoperoxidases have been recently looked for, isolated and purified from 9 coralline algae (Yamada *et al* 1985a) seaweed (Wever *et al* 1985) and from *Pseudomonas aureofaciens* (van Pee and Lingens 1985). Some of the enzymes from marine algae are novel haloperoxidases because they contain vanadium as a prosthetic group: no bromometabolites have been found from fungi (Neidleman and Geigert 1986). However, since halometabolites are known to exist in groups of organisms where no haloperoxidases have been found (e.g. *Moniliaceae*), other sources of halogenating enzymes remain to be discovered. The fact that these organisms are found in a wide variety of habitats explains the widespread presence of halogenated coumpounds.

Thyroid peroxidase, chloroperoxidase, and eosinophil peroxidase have been discovered fairly recently, before 1960, and the bromoperoxidases are only now receiving the same attention (Hewson and Hager 1980; Yamada *et al* 1985b; van Pee and Lingens 1985; Wever *et al* 1985). Even though extensive research has been carried out on halogen containing compounds and the enzymes responsible for their formation, there are many unanswered questions concerning the biosynthesis and function of haloperoxidases and their products. For instance, the step at which CPO catalyzes the chlorination of precursors during in the formation of caldariomycin has not been discovered (Beckwith and Hager 1963), and the function of caldariomycin with respect to *C. fumago* is still unknown.

1.3 *C. fumago* Nomenclature and History

The best known source of CPO is *Caldariomyces fumago*, a sooty mould or a saprophytic, dark pigmented hyphomycete, anamorph class of the Fungi Imperfecti or deuteromycetes. The mould is found commonly on hothouse plants and fallen citrus fruit. The name *Caldariomyces* has been extensively used in the literature since Raistrick and coworkers (Clutterbuck *et al* 1940) first studied the organism. The type species, *Caldariomyces* Woronichin, came from

Woronichin's description in 1926 (Woronichin 1926). However the name used by mycologists is derived from the type species *Leptoxyphium* Spegazinni 1918 (Carmichael *et al* 1980).

Leptoxyphium sp. are characterized by capnodiaceous hyphae with mucilaginous walls. Conidiophores will aggregate to form synnemata (conidiomata) and the conidia produced are minute and hyaline later enlarging and developing pigmented walls and median septa (Hughes 1976).

1.4 CPO Studies

Before 1940, little was known about the biological mechanism or occurrence of halometabolite production. Microbial halogenation was considered a rarity until the 1960's (Petty 1961). This was despite the abundance of inorganic halogen in nature (Neidleman and Geigert 1986) and the fact that Raistrick and co-workers (Clutterbuck *et al* 1940) had looked at 139 species or strains of fungi for the uptake of radioactive chloride and the formation of chlorometabolites and found that almost all the fungi surveyed incorporated inorganic chloride into an organic form (Neidleman 1975).

Chloroperoxidase research has been primarily focused on the study of the biochemical aspects of CPO activity. However recently a physiological study was carried out on CPO production. By sequencing the CPO cDNA and constructing probes for the detection of CPO mRNA, Hager and coworkers (Fang *et al* 1986; Axley *et al* 1986) were able to discover that the observed stimulatory effect of fructose on CPO production (Pickard 1981) works at the mRNA level in CPO synthesis. Besides the above observation, the possible role of CPO in the last chlorination step during caldariomycin biosynthesis (Beckwith and Hager 1963), the elucidation of a defined growth medium (Pickard 1981), and the description of the glucose catabolic pathway of *C. fumago* (Ramachandran and Gottlieb 1963), very little else is known about the physiology of CPO production by *C. fumago*. In fact very little is known about the physiology of fungi in general, with the exception of some yeasts and the industrially important filamentous fungi (e.g. *Aspergillus* sp., *Trichoderma* sp.). Still, interestingly enough, researchers have forged ahead with the development of processes involving fungi since this group of organisms represents a vast pool of proven and potentially useful compounds.

However, for the most part, investigations by Hager and co-workers have concentrated on the biochemical aspects of CPO and were made possible by the pioneering work on the spectral properties of the enzymic intermediates and the kinetics of peroxidases investigated during the 1950's (Chance 1952; Booth and Saunders 1956). Chloroperoxidase was first identified and characterized in the late 1950's (Shaw and Hager 1959a; Shaw and Hager 1959b; Shaw and Hager 1961). In the early 1960's studies were carried out to show the involvement of CPO in the biosynthesis of caldariomycin (Beckwith and Hager 1963), followed by the development of a kinetic enzyme assay (Hager *et al* 1966), and the purification and crystallization and characterization of CPO (Morris and Hager 1966). The Reinheitzahl coefficient (Rz) has been used as an indication of the purity of the enzyme preparation. A Rz greater than 1.4 would indicate the enzyme preparation was pure. Further experiments led to the grouping of chloroperoxidase substrates (Thomas *et al* 1970).

More recently studies have been carried out using elegant techniques to elucidate the nature of the enzyme's active site (Hollenberg and Hager 1973; Krejcarek *et al* 1976; Remba *et al* 1979) and to understand the halogenation reaction mechanism for haloperoxidases (Libby *et al* 1982; Lambeir and Dunford 1983; Geigert *et al* 1983b; Griffin and Ashley 1984; Dunford *et al* 1987). The spectral changes shown by CPO upon the addition of halide salts and hydrogen peroxide were investigated resulting in the proposal of 5 reaction cycles for CPO (Thomas *et al* 1970; Kanofsky 1984; Neidleman and Geigert 1986). The complex nature of the interelationship of the many reaction cycles are shown in Fig. 2. Also the kinetics of cyanide binding to CPO is affected by the presence of nitrate (Lambeir *et al* 1983). The binding of nitrate was shown to be competitive with cyanide.

Three proposed theories exist for the reaction mechanism. The first postulates the presence

Figure 2. Reaction cycles of chloroperoxidase.

(1) Peroxidase cycle

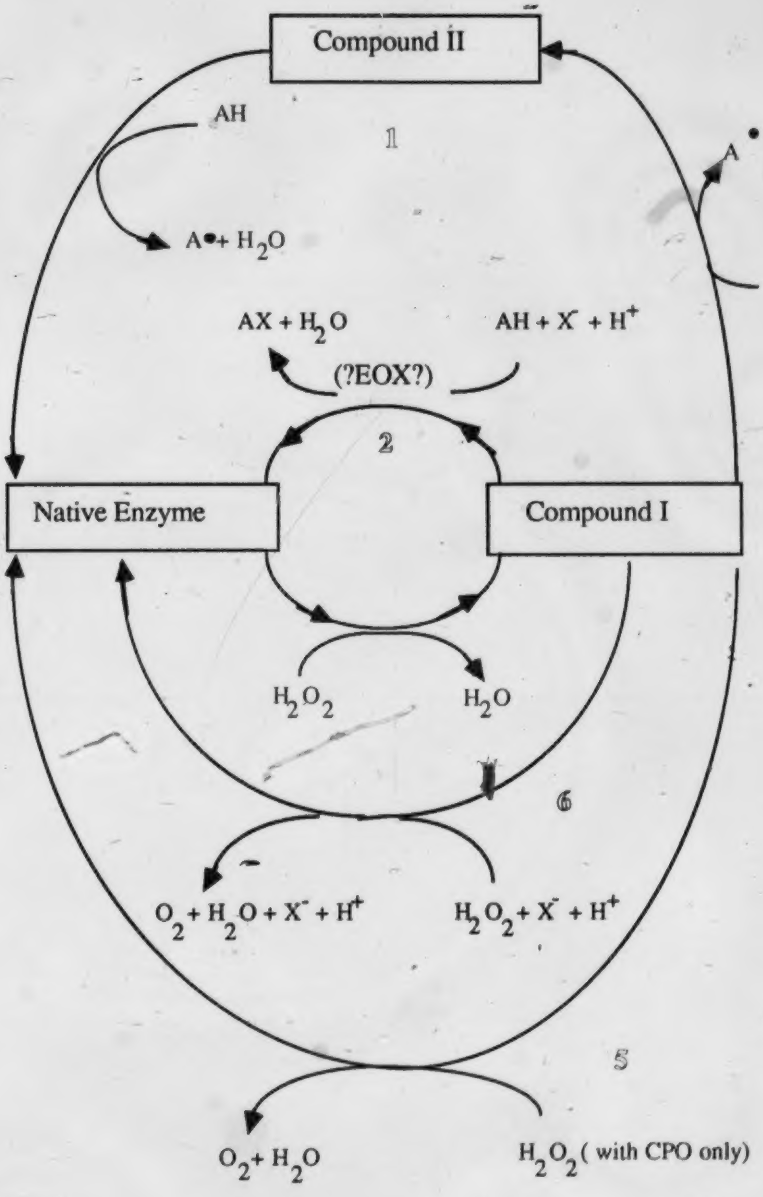
(2) Halogenase cycle

(3) Chlorite cycle (not shown)

(4) Oxidase cycle (not shown)

(5) Catalase cycle

(6) Pseudo-catalase cycle


Compound II
AH
1
A• + H_2O
A•
AH
AX + H_2O
AH + X^- + H^+
(?EOX?)
2
Native Enzyme
Compound I
H_2O_2
H_2O
6
O_2 + H_2O + X^- + H^+
H_2O_2 + X^- + H^+
5
O_2 + H_2O
H_2O_2 (with CPO only)

-from Neidleman and Geigert 1986.

of a diffusable reactive intermediate, HOX (Geigert *et al* 1983; Griffin 1983). The second theory calls for the existence of a enzyme bound chemical intermediate halogenating species, Compound EOX (Lambeir and Dunford 1983; Neidleman and Geigert 1986; Dunford *et al* 1987). The third calls for a mixed enzymatic/nonenzymatic radical chain mechanism (Griffin and Ashley 1984).

Basic research has given way to more applied research in the last few years. Enzyme production and potential applications have been studied by many groups of workers. Pickard (1981) has optimized the growth medium for chloroperoxidase production. Previous researchers (Hollenberg and Hager 1968) had used a glucose-malt extract medium which resulted in high pigment production, pH instability, and as a result, downstream processing problems. However using 10 strains of *C. fumago*, it was found that a fructose-salts medium gave comparable levels of enzyme production in a shorter incubation time, greater pH stability, and the formation of much less pigment. This involved testing the effect of 14 carbohydrates and 15 nitrogen sources with two high producing strains of *C. fumago*. Fructose, which was shown to be the optimum carbon source for CPO production (Pickard 1981), has now been shown to be an inducer of CPO mRNA biosynthesis. Alternatively glucose, a poor carbon source for enzyme production, represses CPO mRNA (Axley *et al* 1986).

Using purified enzyme obtained by a modified purification procedure of Hollenberg and Hager (1978), studies were undertaken to compare enzymes from different strains. Polyacrylamide gel electrophoresis at pH 3 and IEF-PAGE showed isoenzymic forms of chloroperoxidase (Pickard and Hashimoto 1982) confirming previously reported results (Sae and Cunningham 1979). Further studies confirmed the isoenzymes are catalytically identical but contain variable amounts of carbohydrate (Hashimoto and Pickard 1984). Isoenzymes have also been found for HRP (Aibara *et al* 1982) and other haloperoxidases (Neidleman and Geigert 1986). Note that the carbohydrate portion of the enzyme may be exploited during its immobilization (Marek *et al* 1984).

Successful modifications to the purification protocol have been undertaken in order to handle large quantities of enzyme (tangential flow filtration and the adoption of a PEG mediated pigment precipitation (Gonzales-Vergara *et al* 1986) and have aided in the downstream processing of CPO. Longterm CPO stability studies and investigations into the composition of the carbohydrate content of the isoenzymes have also been carried out and await publication (Pickard unpublished data).

Other observations include the ability of CPO to inactivate amylases (Mitchell *et al* 1981) and to catalyze the oxidation of iodine to iodate (Thomas and Hager 1968), form nitroso compounds from amino groups (Corbett *et al* 1980), and the dismutation of chlorine dioxide (Shahangian and Hager 1981). It will also oxidize primary alcohols to aldehydes (Geigert *et al* 1983b), DMSO to dimethyl sulfone (Geigert *et al* 1983c) and alkenes to epoxides (Geigert *et al* 1986). Other than the amylase inactivation, all of these reactions are unique to CPO among the haloperoxidases. Recently CPO has been used for the bioelectrosynthesis of halogenated barbiturates (Laane *et al* 1986).

It has been reported that the heme portion of the enzyme is not absolutely required for CPO activity (Geigert and Neidleman 1985; Liu *et al* 1987). Other heme-less peroxidases isolated include non-heme bromoperoxidase and lactoperoxidase and glutathione peroxidase (Dumontet 1983; Neidleman and Geigert 1986). Enzymes containing manganese and vanadium have been found and a variety of metal and porphyrin substituted 'synthetic' haloperoxidases have been prepared and studied (Neidleman and Geigert 1986).

Recently, CPO cDNA has been cloned and sequenced. The primary sequence codes for a 32,974 dalton, 300 amino acid mature protein and a 21 amino acid signal peptide (Fang *et al* 1986) which undergoes a number of post-translational modifications. The modifications include deaminations at Asn12, Asn198, and Gln183, two N-glycosylation sites, and the cyclizing of a glutamic acid into pyroglutamic acid (Kenigsberg *et al* 1987).

1.5 Potential Applications of Cells and Enzymes

Throughout history man had taken advantage of the metabolic strength and diversity of free microbes and immobilized cells to improve their quality of life (e.g. bread, beer and wine making). With the advent of the concept of cell immobilization and the use of immobilized cells in cell dependent processes (Tosa *et al* 1979) and all the developments necessary for the many prospective applications of novel microbial processes, man's microbial improvisation has grown into industrial microbiology, a prevalent part of the world economy accounting for tens of billions of dollars annually in the U.S. alone (Demain and Solomon 1981).

During the last 15 years, many immobilized cell techniques and applications for bacteria and then fungi have been explored and presented by researchers. Initially experiments were confined to the use of immobilized cells as biocatalysts for the production of low molecular weight compounds. In many instances this involved the immobilization of non-viable whole cells. The enzymes, still active inside, are used to carry out simple biotransformations such as amino acid production (Sato *et al* 1979) which required no cofactors. The next stage in research involved the use of viable immobilized cells, which carry out multistep reactions that utilized a number of enzymes and cofactors, to synthesize low molecular weight compounds like ethanol. Finally, the production of macromolecules (enzymes) by immobilized cells was attempted on a limited scale. Some examples include the use of acrylamide entrapped *Bacillus subtilis* and *Streptomyces fradiae* for the production of amylase (Kokubu *et al* 1978), and protease (Kokubu *et al* 1981) respectively, *Trichoderma reesei* immobilized in carrageenan (Frein *et al* 1982) for cellulase production and agarose immobilized *Phanerochaete chrysosporium* for the continuous production of lignin peroxidase (Linko *et al* 1986). However, despite all the effort and optimism, very few immobilized cell systems have been applied on an industrial scale (Anderson 1983).

Today, there are two major commercial applications for microbial cells: as a source of protein and as a biocatalyst in chemical transformations. Enzymes have only been used in commercial processes that are primarily degradative in nature (Lambert 1983). The recent successful exploitation of enzymes has resulted in competition and conflict in the race to find new applications for microbial cells and enzymes. For instance, the use of immobilized viable and permeabilized cells has been suggested as an alternative to immobilized enzyme systems because the costly need for purification would be eliminated. Yet enzymes are ultimately responsible for all biotransformations and therefore the potential bypassing of microbes exists for processes requiring specific biochemical conversions.

Thus, in the 1960's and 1970's, there was excitement and optimism associated with research on 'enzyme technology', which exploited the knowledge and methodology of many branches of science. Commercial scale enzyme production facilities eventually arose from this research and by 1985 the world market for bulk industrial enzymes was estimated to be 500 million dollars U.S.(Lambert 1983). This research also resulted in a wide variety of potential applications proposed for enzymes involving many methods of preparation of free or immobilized enzymes. A few examples of these uses are the coimmobilization of lactoperoxidase and glucose oxidase by entrapment in κ-carrageenan in order to produce continuously alkene halohydrins (Kawakami 1986) and the use of partially purified enzymes for the synthesis of β-lactam antibiotics (Jensen *et al* 1984). However, as was the case with immobilized cells, scientific optimism has not led directly to many industrial applications for enzymes. Only a few enzymes have been implemented in large-scale processes (e.g. glucose isomerase for high fructose syrup production, proteases in detergents, amino-acylase in penicillin derivative production). Despite their potential, enzymes have some important limitations, such as their relative fragility when compared to other industrial catalysts (Witholt *et al* 1985), which has slowed their acceptance and use in industry. Yet problems such as enzyme instability may be overcome in the near future if advances in protein engineering lead to the production of modified, stabilized enzymes (Witholt *et al* 1985) and semi-synthetic catalysts (Lee 1984).

Research into the potential use of macromolecules and microorganisms in industry often assumes there will be a large demand for the use of enzymes (Wolfe *et al* 1984; Alberti and Klibanov 1981; Geigert and Neidleman 1985) or microbes (Livernoche *et al* 1983; Messing 1982) or their metabolites (Chibata *et al* 1983). For instance, the elucidation of the requirements for the large scale production of ligninases (Jager *et al* 1985) and cellulases (Frein *et al* 1982, Wase *et al* 1985) would allow for the development of waste conversion techniques in the pulp and paper industry. These assumptions presuppose the idea that industrial firms are open to changing existing methods of production, which are often complex and delicate, or that there are entrepreneurs who will fund the development of new industries. Neither is likely to occur based solely on 'just a good idea' but rather on what is practical and makes sound economical sense (Bucke 1983; Gaden 1981). However, this belief in future demand does allow the researcher to undertake applied research and solve specific problems or investigate alternative methods for existing processes.

1.5.1 Wastewater Treatment

This concept of applied research forms the rationale for this thesis, which is concerned with the production and use of chloroperoxidase for phenolic compound transformations. It was the discovery of the ability of haloperoxidases to oxidize and precipitate a wide variety of phenolic compound compounds present in industrial wastewaters along with the developments in cell/enzyme technology that set the direction of this thesis project. It was decided that if CPO was to be a potential candidate for industrial use it would have to be produced efficiently in large quantities and be cost-effective when compared to existing systems.

Since the production and use of a fungal enzyme is the essence of this project it would be important and relevant to discuss why enzymes are being considered for wastewater treatment and the concerns of fungal enzyme production and application to industrial processes. The interest in developing alternative methods of wastewater treatment is due to the increasing industrial demands placed on conventional systems of treatment and their sometimes apparent inability to prevent the discharge of toxic industrial products into the biosphere. In the past, practices such as the reliance on the dilution and natural treatment capacities of rivers and the use of aerobic biological methods have been acceptable but new industries have created new treatment challenges and the formation of specialized treatment techniques (Manner 1977; van den Heuval *et al* 1981; Demain and Solomon 1981).

In particular the problems presented by phenolic wastewaters have been of much concern. In 1983, it has been estimated that 2.6 billion tons of phenol were produced in the U.S. from synthetic chemical processes (Webber 1984) and natural sources. Petroleum refineries, coke operations and coal conversion processes produce effluents with high concentrations of phenolic compound compounds (Fedorak and Hrudey 1986) which, if released untreated, would pose a serious environmental hazard (Babich and Davis 1981). Conventional treatment systems in use today include many aerobic microbial processes which are both rapid and efficient. Much is known about the limitations of these systems (Neufeld 1984) and the biochemistry involved has been extensively reviewed (Chapman 1972).

In order to meet special and specific problems presented by new industries, researchers are developing alternative wastewater treatment systems. An example would be the treatment of coal conversion plant effluent containing high concentrations of phenolic compound compounds by anaerobic bacterial cultures (Fedorak and Hrudey 1984b). Thus, in addition to the proposed enzyme system, scientists have looked at a variety of alternative methods such as anaerobic treatment (Fedorak, 1984; Fedorak and Hrudey 1984a; Fedorak and Hrudey 1984b) resulting in methane production (Fedorak and Hrudey 1986), modified anaerobic methods including activated carbon reactors (Khan *et al* 1981), and immobilized cells (Anselmo *et al* 1985) in bioreactors (Edeline *et al* 1986; Westmeier and Rehm 1987). Pollution control by enzymes is just one of many alternative methods being considered. The differences between proposed alternative treatment systems has not been evaluated.

1.5.2 Enzyme Catalyzed Phenol Oxidation

The application of enzymes to industrial processes has shown particular promise where degradative, biological systems are already in use (Kierstan and Bucke 1977; Messing 1982). Conventional wastewater treatment systems are relatively simple in nature and therefore become a potential candidate for replacement by enzyme mediated systems (Chapsal *et al* 1986). Enzymic treatment offers many advantages over existing systems including wider temperature spectrum and rapidity of treatment. The potential for enzyme immobilization by one of many techniques would allow for the recovery and reuse of the catalyst. In the case of haloperoxidases, there is also the advantage of broad substrate spectrum and stability (Geigert and Neidleman 1985).

The enzymic treatment of high strength industrial wastewaters has been proposed using HRP (Alberti and Klibanov 1981; Klibanov and Morris 1981), polyphenol oxidase (PPO) (Klibanov *et al* 1983), CPO (this thesis), haemoglobin (Chapsal *et al* 1986), and fungal laccase (FL)(Shuttleworth and Bollag 1986). Enzymes have been used to oxidize a wide variety of aromatic compounds including phenolic compound compounds, halogenated phenols and aromatic amines. Also the use of enzymes has been put forward for the decolourization of pulp effluents (Paice and Jurasek 1984) and the transformation of trace organics from drinking water (Maloney *et al* 1986). However before the latter could be put into effect, the nature of the reaction products must be thoroughly understood. Even though HRP and CPO have long been known to oxidize phenolic compound compounds such as *p*-cresol and tyrosine (Westerfeld and Lowe 1942; Hager *et al* 1966), the extent of this activity had not been considered until recently (Alberti and Klibanov 1981).

A major consideration for the successful application of any enzymes to industrial processes is the scale up of their production (Linko and Linko 1984; Jager *et al* 1985). This includes studies on the development of efficient fermentations resulting in the production of large quantities of active enzyme. These studies would be based on three criteria: (1) the choice of rich enzyme source suited to growth in fermenters (2) the determination of optimum growth conditions (3) the choice of a cheap, defined medium that will cut costs and aid in the development of a computer model for fermentation monitoring and control. These studies will result in the development of a microbial process which maximizes product yield, product concentration and the rate of product formation (Pirt 1975). Many such studies have been carried out over the last 20 years especially in the areas of cell immobilization (Linko and Linko 1984) and extracellular enzyme production (Kubuku *et al* 1981; Frein *et al* 1982).

Wasterwater treatment applications will require purified enzyme in kilogram quantities. Regardless of the activity and longevity of the enzyme and apart from the capital investment on facilities, the production and purification of the enzyme would likely be the most expensive component of such research or industrial process (Messing 1982; Neidleman and Geigert 1986). Thus any method of producing cheaper enzyme would be advantageous. Successful application of enzymes to industrial processes requires that the enzyme compete economically with the system that is currently operational. In the case of the proposed HRP treatment it has been shown to compare favourably with existing methods of removing phenolic compound compounds from wastewaters: it gave comparable removal efficiencies and was cost-effective (Alberti and Klibanov 1981). CPO cost-effectiveness will be dealt with later in the discussion section of this thesis.

1.5.3 Mycelial Pellets and Immobilized Hyphae

The physical characteristics of fungal stirred tank and stationary cultures are different from those of bacteria and yeast (Burkholder and Sinnott 1945). This is due to the morphology of molds, and to the non-Newtonian properties (uneven mixing) the culture exhibits in the filamentous form. The high viscosity of the filamentous fungal cultures results in mass transfer, mixing and heat transfer problems, which may be overcome by changing engineering variables (van Suijdam and Metz 1981). For instance, an increase in power input to the impeller would lead to reduced hyphal length, thereby reducing viscosity. However the amount of energy input would be dependent on the tensile strength and age of the hyphae and would obey the law of diminishing returns.

Industrial procedures for fungal enzyme production vary to a degree (i.e.bioreactor design) but all can be classified as either solid-state or submerged fermentation. In the case of the latter both batch and fed-batch processes are the most widely used. Also, as mentioned earlier, semi-continuous and continuous culture have been used, as in the successful production of proteases (Jensen 1972), but these procedures have yet to be tried in a commercial system (Lambert 1983).

In the form of mycelial pellets or immobilized hyphae, fungal cultures offer several advantages over loose mycelial cultures, the most important being a decrease in viscosity. Others include improved activity yield and biocatalyst stability, potentially easier downstream processing and manipulation of the immobilized cells. However the use of fungal pellets for fermentations can cause problems by decreasing effective oxygen diffusibility and hamper sampling during growth studies.

The use of mycelial pellets in stirred tank and airlift cultures has been reported (Martin and Waters 1952; Steel *et al* 1954; Clark 1962; Konig *et al* 1982; Kloosterman and Lilly 1985; Martin and Bailey 1985; Wase *et al* 1985) and much is known about the factors affecting pellet formation (Ward and Colotelo 1960; Whitaker and Long 1973; van Suijdam *et al* 1980; Schurgerl *et al* 1983). Some of the factors are agitation, growth medium, pH, oxygen tension, polymer additives, and growth rate. The size of inoculum has been recognized as of great importance in pellet formation. High inocula generally result in filamentous growth while low inocula produce mycelial pellets (Metz and Kossen 1977).

Mycelial pellets have also been examined for oxygen mass transfer (Ho *et al* 1984) to show that oxygen depletion in the pellet or bead center will cause autolysis of the fungus resulting in a hollow center (Metz and Kossen 1977). Also immobilized hyphae have been examined microscopically to show that the degree of mycelial growth inside the beads was affected by the κ-carrageenan concentration (Deo *et al* 1983). A concentration of 4% κ-carrageenan resulted in peripheral growth.

Directed pelletizations or immobilized fungal cells have been reported in the literature for a wide variety of applications including the production of citric acid (Tsay and To 1987), enzymes (Kobuku *et al* 1978; Kobuku *et al* 1981; Frein *et al* 1982; Wase *et al* 1985; Suter *et al* 1986; Kirk *et al* 1986), ethanol (Linko *et al* 1981; AiresBarros *et al* 1987; Godia *et al* 1987), gibberellic acid (Kahlon and Malhotra 1986), gluconate (Heinrich and Rehm 1982), glutathione (Murata *et al* 1981), glycerol (Bisping and Rehm 1982), itaconic acid (Horitsu *et al* 1983; Ju and Wang 1986), and the decolourization of mill effluents (Livernoche *et al* 1983). Itaconic acid (Ju and Wang 1986), and ethanol (Godia *et al* 1987) have been produced continuously. More recently researchers have begun using genetically modified bacterial and fungal cells to produce extracellular foreign proteins (Das and Shultz 1987; Younes *et al* 1987).

1.6 Thesis Objective and Rationale

CPO, an extracellular fungal enzyme, can be readily derived from the deuteromycete *Caldariomyces fumago* when grown in a defined medium that supports good growth and enzyme production (Pickard, 1981). In view of the recent increase and advantages in the use of immobilized cells for the production of low molecular weight compounds (Linko and Linko 1984), enzymes (Frein *et al* 1982) and antibiotics (Jones *et al* 1983), it was of interest to determine if CPO, which can be routinely produced by free mycelia in shake flask cultures, could also be produced by mycelial pellets or immobilized hyphae in a semi-continuous or continuous process in small scale fermenters.

This thesis describes some of the experiments carried out on the semi-continuous production of CPO by *C. fumago* immobilized in κ-carrageenan and continuous production of CPO by free pellets and immobilized cells in a tower-loop airlift fermenter. Also investigated was the ability of free pellets of *C. fumago* to produce CPO in a pilot plant scale 50 L fermenter. Ultimately purified enzyme was used to oxidize a variety of phenolic compound compounds and immobilized by entrapment in κ-carrageenan for the treatment of synthetic phenolic wastewaters.

2. METHODS AND MATERIALS

2.1 Microbiological

Caldariomyces fumago ATCC 16373 and *C. fumago* ATCC 11925 were obtained from the American Type Culture Collection, Rockville, MD., U.S.A. and *C. fumago* CMI 89362 was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England. The Biosystematics Research Institute, Agriculture Canada, Ottawa supplied the following cultures: *C. fumago* DOAM 145537, *Caldariomyces* sp. strains DOAM 138163, DOAM 119795, DAOM 137632, *Leptoxyphium axillatum* DOAM 167770, and *Leptoxyphium* sp. strains DOAM 166438 and DOAM 165359. The strains were maintained on potato dextrose agar plates at 4 °C and were transferred to fresh plates every 6 months. The six DOAM strains have been deposited in the UAMH under the care of L. Sigler.

2.2 Stock Culture Preparation

Since *C. fumago* does not readily sporulate, stock cultures were initiated by excising a 1 cm^2 piece of mycelia mat from an agar plate into 50 mL of phytone-fructose salts medium (PFS), containing (g/L): phytone (BBL Phytone peptone), 20; fructose, 40; $NaNO_3$, 2; KCl, 2; KH_2PO_4, 2; $MgSO_4 \cdot 7H_2O$, 1 and $FeSO_4 \cdot 7H_2O$, 0.02. The mycelium was homogenized for 15 s at full speed in a Sorvall Omnimixer and the resulting fragments grown in a 125 mL Erlenmeyer flask for 5 days at 27 °C on a rotary shaker at 200 rpm. The resulting growth was streaked onto cereal agar (MeadJohnson, Bristol-Myers) or PDA (Difco) and incubated at 27 °C until good growth had occurred. These plates were used as working stocks for future growth experiments.

Starter cultures also were begun by excising mycelium from the stock agar plate into 50 mL or 200 mL of PFS. The mycelium and 50 mL of PFS medium were homogenized and the resulting fragments were grown as above. Fructose salts (FS) medium, the same as PFS but without the phytone (Pickard 1981), was also used for growth of the fungus and enzyme production (200 mL/500mL Erlenmyer flask).

2.3 Inoculum Preparation

Some degree of inoculum standardization was achieved by determining the appropriate wet weight of mycelium, which would give the best enzyme production when inoculated into 200 mL PFS medium in a 500 mL Erlenmeyer flask. It was found that 400 mg wet weight homogenized mycelium, from a mycelial mat or starter culture gave reproducibly high levels of enzyme production after 7-10 days growth.

2.4 Pellet Formation

Control of the inoculum concentration in inoculum cultures of *C. fumago*, 50-100 mg wet weight homogenized mycelium in 200 mL PFS, resulted in the formation of small (approximately 3 mm diameter) fungal pellets after 1 week incubation at 28 °C and 200 rpm in a 500 mL flask.

2.5 Immobilization

After 5 days of growth of an inoculum culture, the chloroperoxidase level in the medium approached values of 50 - 100 mg/L, depending on the strain of *C. fumago* employed (Hashimoto and Pickard 1984). Cultures were usually particulate and required a further homogenization step, as described above. Hyphal fragments had to be small enough to pass through a 20 gauge syringe as described below. The homogenized mycelium was immobilized in 4% κ-carrageenan (NJAL 798, Marine Colloids Division, FMC Corporation Inc., Rockland, Maine) as described previously (Tosa *et al* 1979): homogenized culture (10 mL, approximately 100 mg wet weight mycelium) was added to sterile 5% (w/v) aqueous κ-carrageenan (40 mL) and the mixture stirred until the dark mycelial particles were evenly distributed. The mixture was then extruded dropwise from a sterile 50 mL plastic syringe fitted with a 20-gauge needle into 200 volumes of sterile hardening solution (0.3 M KCl + 0.01 M $CaCl_2$) at 4 °C. The resultant gel beads (3 mm diameter) were gently stirred for 1 h then washed three times by aspiration with 1 L sterile distilled water. Aspiration was carried out with a wide-mouthed 10 mL pipette on a vacuum line.

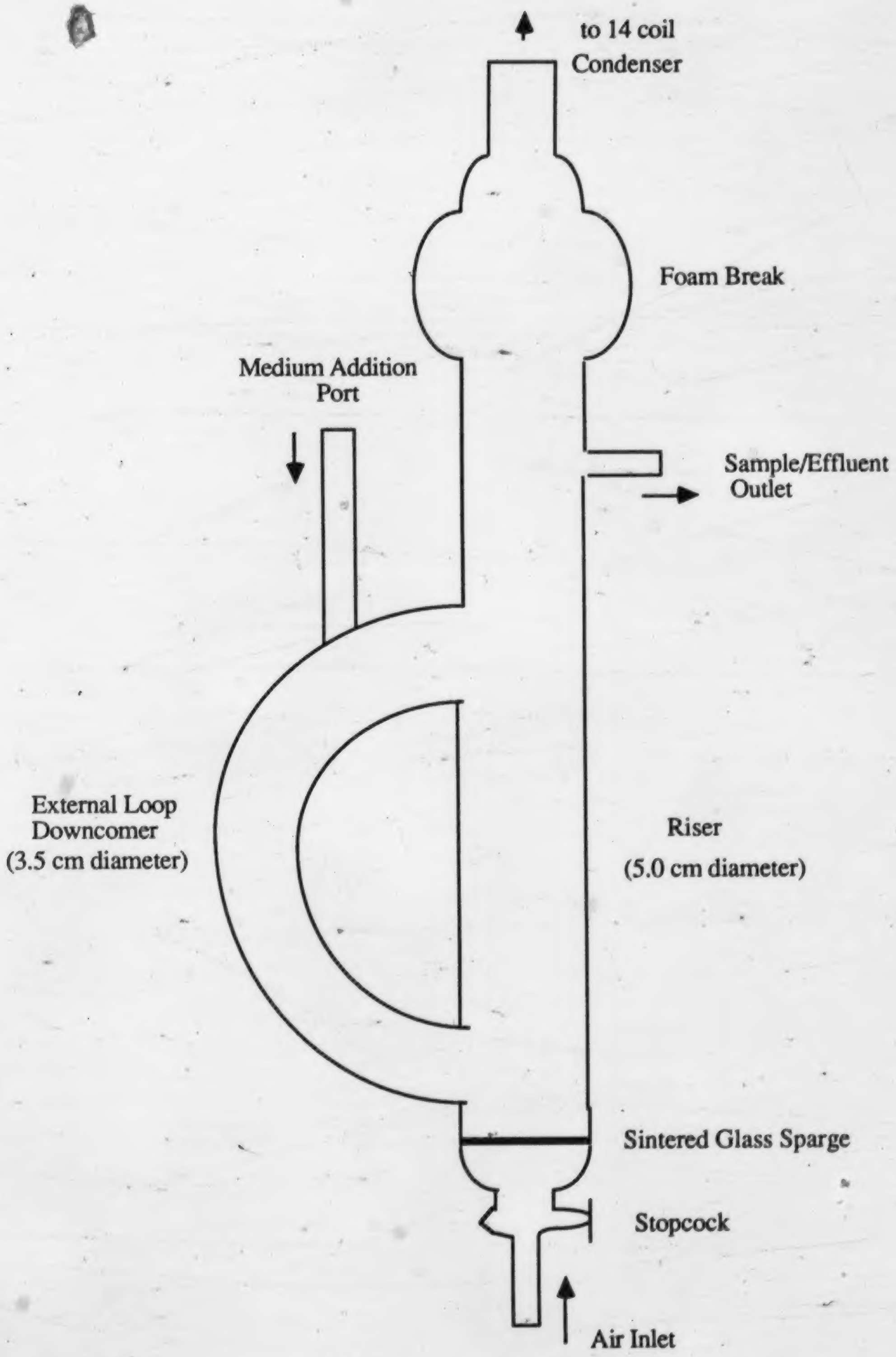
2.6 Semi-Continuous Production of Enzyme

About 20 mL of beads (3 mm diameter), prepared as described above, were transferred to 200 mL PFS in a 500 mL Erlenmeyer flask and incubated a further 5 days with shaking at 27 °C. By this point the beads had turned black due to vigorous mycelial growth and high levels of enzyme were present in the culture supernatant. The complex medium was removed by aspiration, the beads washed with 20 mM potassium phosphate buffer, pH 5.5, and the medium changed to the defined FS formulation (pH 4.0). Semi-continuous enzyme production by immobilized *C. fumago* was achieved by repeated transfers of the fungal beads to fresh FS medium after washing with the buffer described above. Cultures were transferred prior to the pH rising above 6.0, which caused irreversible enzyme denaturation.

2.7 Airlift Fermenter Culture

The inoculum for fermenter cultures was *C. fumago* CMI 89362 grown either in the form of pellets for <1 week (pellet size = 1-3 mm diameter) in PFS or FS medium or immobilized in κ-carrageenan as described earlier. Growth and enzyme production was carried out in an airlift fermenter (height/diameter ratio = 10/1, operating volume 1.0 L, sintered glass sparger) with an external loop as shown in Fig. 1. A BRL model 2200AF airlift fermenter (1.8 L working volume) with an internal baffle loop (3.5" O.D. x 3.12" I.D.x 3/16" wall) equipped for air, pH, temperature and foaming control was also used for some experiments. Cultures were incubated at 22 °C and sparged with enough air to keep the pellets or beads circulating (0.6-1.0 L/min, 0.2 μm filter sterilized air). The air was prescrubbed with glass wool and calcium chloride. Samples were taken from a silicone tubing line which protruded into the downcomer loop of the fermenter.

Figure 1. Tower external loop airlift fermenter.


to 14 coil
Condenser
Foam Break
Medium Addition
Port
Sample/Effluent
Outlet
External Loop
Downcomer
(3.5 cm diameter)
Riser
(5.0 cm diameter)
Sintered Glass Sparge
Stopcock
Air Inlet

2.8 Continuous Production of Enzyme

Continuous production of CPO was carried out in the airlift fermenter with an external loop as described above. Later studies used a modified version of the above described tower external loop airlift fermenter with a foam break and short coil condenser (dimensions same as above). Fermenters were inoculated with a measured settled volume of immobilized hyphae or pellets (usually 30-50 mL or approximately 300 pellets/fermenter). The medium in the fermenter was varied and the pellets were circulated (pellet circulation time = 5 s, aeration rate = 0.6 L/min) without addition or removal of medium until the CPO level in the culture supernatant had reached an elevated level. At this point, spent medium was removed and fresh medium added at the same rate using a 2-channel peristaltic pump. As the pellets grew, some were removed in order to maintain the fluidity of the culture in the fermenter while the remaining pellets were saved for the next run. The inoculum for the continuously-fed airlift fermenter cultures could be continually transferred and used as long as it produced high enzyme levels (>250 EU/mL). In one case the pellets were used in 6 fermenter cultures.

Samples were centrifuged (3000xg, 10 min) and supernatants were stored at -20 °C. Enzyme, pH, sugar, nitrate and pigment concentrations were determined ~~on the~~ supernatants.

2.9 Large Scale Production Of CPO

Large scale production of CPO by *C. fumago* pellets was accomplished using a LH 2000 Series Fermenter with temperature, and pH PID (proportional, intergral, derivative) type controllers and with a pO_2 proportional type controller. The fermenter has a height/diameter ratio of 3/1 and an operating volume of 50 L. Aeration was set at 30 L/min. Temperature was maintained at 27 ± 0.5 °C and the agitation speed was 100 rpm. A three step inoculum preparation procedure was used to bring about pellet formation. Starter and sub-cultures were initiated as described earlier. The small pellets obtained from the subculture were homogenized and 60 mg each was transferred into 5 flasks of 200 mL PFS and grown for 8 days at 27 °C. The medium was then decanted and the mycelium was homogenized in 50 mL FS medium. From this third subculture, 22 g wet weight mycelium was used to inoculate the fermenter containing 50 L FS medium. Samples were taken and assayed for enzyme and pigment concentration. After 26 days, as CPO levels increased, the supernatant was separated from the fungal pellets by filtration through nylon stockings and frozen before enzyme purification. Harvesting was forced at this time due to a steam shutdown.

2.10 CPO Purification

CPO was purified from *Caldariomyces fumago* CMI 89362 as described by Pickard and Hashimoto (1982) except that the initial concentration of the fungal growth medium and dialysis was carried out using a Millipore Pellicon tangential flow cassette system (1.0 ft^2 10K cutoff). After ethanol precipitation and column chromatography through DE-52 and Sephadex P-60, the preparation, used for later phenolic oxidation studies, had a Rz >1.4 (Morris and Hager 1966).

2.11 Enzyme Assay

Enzyme activity was determined kinetically by measuring the chlorinating activity of CPO, as described previously (Pickard 1981). Enzyme activities are expressed as mg/L based on a specific activity of crystalline CPO of 1660 µmoles/min/ mg (Morris and Hager 1966; Hashimoto and Pickard 1984). Reactions were performed with approximately 3 ng of CPO in a 1 mL reaction mixture.

2.12 Phenolic Oxidation

Halide-independent peroxidation of phenolic compounds was determined by following the removal of test compounds from the reaction mixture by gas chromatographic analysis. In the survey studies, reaction mixtures initially contained 200 µg phenolic and 0.2 µmol H_2O_2 in 2 mL 0.1 M potassium phosphate-H_3PO_4 buffers at pH 3 and 5.5. In experiments employing phenol, molecular weight of 94 g/mol, this corresponded to approximately 1 µmol substrate in each 1 mL reaction. Reactions were started by the addition of 5 µg purified CPO to one-half of the mixtures. All the reactions were carried out at 22 °C. For the survey experiments 3 further additions of CPO $+H_2O_2$ (experimental) and H_2O_2 alone were made at 30 min intervals.

Samples (5 µL) were taken from the reaction and no enzyme control mixtures before each peroxide addition and at the end of 2 h and injected directly (injector temperature= 230 °C) into a Hewlett-Packard model 5790 gas chromatograph (GC) equipped with a flame ionization detector (set at 250 °C). The stainless steel column (2m X 2mm) was packed with Tenax-GC (60-80 mesh) coated with polyphenyl ether (5%). Samples were run isothermally at 200 °C using a nitrogen carrier gas (30 mL/min). Peak areas were determined using a Hewlett-Packard model 3390A integrator and compared with standard curves.

2.13 Phenolic Wastewaters

Two wastewaters were obtained from Dr. P.M. Fedorak, Dept. of Microbiology, U. of A., and treated with CPO and hydrogen peroxide. The coal coking wastewater was from a steel plant and contained 410 mg/L phenolics (Fedorak and Hrudey 1987). The other wastewater (7600 mg/L phenolics) was from a H-coal conversion pilot plant process (Fedorak and Hrudey 1984b).

2.14 Estimation of Enzyme Stability

The enzyme is most active as a chlorinating enzyme at pH 3 and thus the spectrophotometric monochlorodemidone assay method of Morris and Hager (1966) was used to monitor any changes in enzyme stability. Enzyme plus substrate were incubated in 0.1 M potassium phosphate-H_3PO_4 buffer, pH 5.5 and 1-50µL aliquots were removed, diluted with 0.1M potassium phosphate-H_3PO_4 buffer, pH 2.75 and assayed for chlorinating activity. The effect of pH on phenol oxidation was tested over a range from 2.0-9.5 using 0.1 M phosphate buffers.

2.15 Enzyme Immobilization

Purified CPO (Rz.1.4, Specific activity=1600EU/mg) was immobilized by using a 5% glutaraldehyde aggregation step at 4 °C followed by entrapment in 5% κ-carrageenan. Purified enzyme (4 mg in 480 μL) was pretreated with 70% glutaraldehyde (20 μL) for 30 min and then mixed with 9.5 mL of 5% κ-carrageenan. Immobilization resulted in the formation of 8 mL volume of settled immobilized enzyme gel. The gel-entrapment method used was the same as the cell immobilization protocol described earlier, except that the hardening solution contained 3.5 % glutaraldehyde. The immobilized enzyme reactor used was a modified version of the one described by Jensen *et al* (1984), where a pasteur pipette was substituted for the tuberulin syringe and a 50 mL beaker was used as a reaction mixture vessel instead of a test tube.

2.16 Analytical Methods

Samples were centrifuged (3000xg, 10 min) prior to determining pH values and sugar content. Measurements of pH were carried out using a Fisher Accumet model 230 meter and Fisher (Ingold) combination probe calibrated to pH 4.0. Pigment levels were monitored at 575 nm. Potassium phosphate-H_3PO_4 buffer (20 mM, pH 5.5) was used as diluent and blank. Fructose levels remaining in the culture supernatant were determined using a total carbohydrate assay (Dubois *et al* 1956).

Nitrate levels were determined analytically by reduction to nitric oxide. The nitric oxide was measured through its chemiluminescence reaction with ozone using a Chemiluminescent NO_x Analyzer (Yoshizumi *et al* 1985), courtesy of Dr. Monica Palcic, Dept. of Food Science, U. of A.

Spectra were obtained using a Pye-Unicam model SP8-100 ultraviolet spectophotometer using 0.1 M potassium phosphate-H_3PO_4 buffer, pH 3.0 as diluent and blank.

The wet weight of the homogenized mycelium used for inoculations was determined rapidly using vacuum filtration (10 min) through Whatman No. 1 filter paper.

Fungal density (dry weight/unit volume) was determined by vacuum filtration of the culture medium through Schleicher and Schuell #520B 15 cm filter circles and measurement of the culture filtrate volume. The collected mycelia were washed with distilled water and the mycelia were dried to a constant weight at 110 °C (approximately 12 h). In most cases the total number of fungal pellets/culture were determined by a direct count and the dry weight of fungus in a pellet was calculated using the total biomass recovered from the fermenter.

2.17 Chemicals

Hydrogen peroxide was obtained from Amachem (30% solution) and peracetic acid from Terochem (40% solution). κ-Carrageenan was a gift from FMC Marine Colloids Division under the name of Gelcarin c/c 798. β-D(-)Fructose was from Sigma and 'BBL Phytone' peptone (papaic digest of soybean meal) was from Becton Dickinson and Company. The phenolic compounds used in this study were made by Aldrich Chemical Company, Inc. and were kindly supplied by Dr. P.M. Fedorak. All inorganic chemicals used were of reagent grade.

3. RESULTS

The results section of this thesis is divided into two parts. The first part (3.1) is concerned with chloroperoxidase production in shake flask and fermenter cultures by immobilized hyphae and pellets of *C. fumago*. The second section (3.2) is devoted to the phenolic transformations catalyzed by the halide-independent peroxidase activity of chloroperoxidase.

3.1 CHLOROPEROXIDASE PRODUCTION STUDIES

Pickard and workers have been able to devise the methodology for the preparation of CPO from shake flask culture for the production of gram quantities of enzyme. However this method is somewhat unsatisfactory due to the amount of labor required to grow the fungus on a large scale. Also, from time to time, a batch culture of *C. fumago* failed to produce the expected levels of CPO and this failure was associated with the deterioration of the culture to a highly viscous fungal slurry which severely hampered enzyme purification. Therefore experiments were designed to investigate alternative methods of culture with the hope of improving the consistency and cost effectiveness of enzyme production and at the same time gain some insight into the physiology of the fungus.

3.1.1 Strain Selection

The first experiments carried out were designed to select high CPO producing strains of *Caldariomyces* or *Leptoxyphium* suitable for later immobilization studies. Table 1 shows the maximum enzyme levels achieved by 10 strains of *Caldariomyces* sp.and *Leptoxyphium* sp. and the morphology they exhibited in two successive PFS cultures. Two cultures, ATCC 11925 and CMI 89362, were chosen for immobilization for their high enzyme production (>100 mg/L) and 'convenient morphology' while a third strain (DOAM 137632) was selected to determine if immobilization would have a positive effect on a low enzyme producing strain.

Table 1. Strain Survey for Enzyme Production and Culture Morphology in Phytone Fructose Salts Medium.

	CPO Activity (mg/L)		
Strain #	Transfer 1	Transfer 2	Morphology
DOAM 138163	14	25	mycelial slurry, very viscous, high pigment
DOAM 165359	11	40	mycelial slurry, very viscous, high pigment
DOAM 166438	31	76	small pellets, slightly viscous, high pigment
DOAM 145537	16	53	large pellets, slightly viscous, high pigment
DOAM 137632	40	62	large pellets, slightly viscous, high pigment
DOAM 167770	24	8.4	large pellets, low viscosity, high pigment
DOAM 119795	33	59	mycelial slurry, very viscous, high pigment
ATCC 11925	170	160	small pellets, low viscosity, low pigment
ATCC 16373	8.4	13	mycelial slurry, high viscosity, high pigment
CMI 89362	130	180	large pellets, low viscosity, medium pigment

C. fumago strains were excised from PDA plates and homogenized in growth medium. A 2% inoculum of each strain was transferred to 50 mL PFS and grown as described the methods section. Cultures were monitored for culture morphology.

Cultures were considered convenient if they grew in large firm pellets, with little hyphal shedding, produced little pigment and maintained low viscosity. Note that conditions for the formation of pellets were not optimized for each strain.

3.1.2 Effect of Free Hyphal Inoculum Size on Culture Morpholgy and Enzyme Production

Throughout this study it was apparent that the method of preparation of the inocula for shake flask and fermenter cultures was of some importance. The effect of inoculum size on enzyme production levels and the time taken to reach maximum enzyme levels were studied using shake flask cultures of *C. fumago* CMI 89362 in PFS medium (see Fig. 4). Large inocula resulted in a more rapid accumulation of CPO in the culture supernatant. However the final levels attained (120 mg CPO/L) were the same as low inoculum cultures. At the same time the mycelial morphology was observed and later experiments showed that small inocula (<100 mg/200 mL PFS) gave rise to pellets whereas high inocula (>200 mg/200 mL PFS) resulted in thick mycelial slurries after 1 week's growth at 28 °C and 250 rpm. These pellets were used for later comparison with the immobilized hyphae system.

3.1.3 CPO Production by Immobilized *C. fumago*

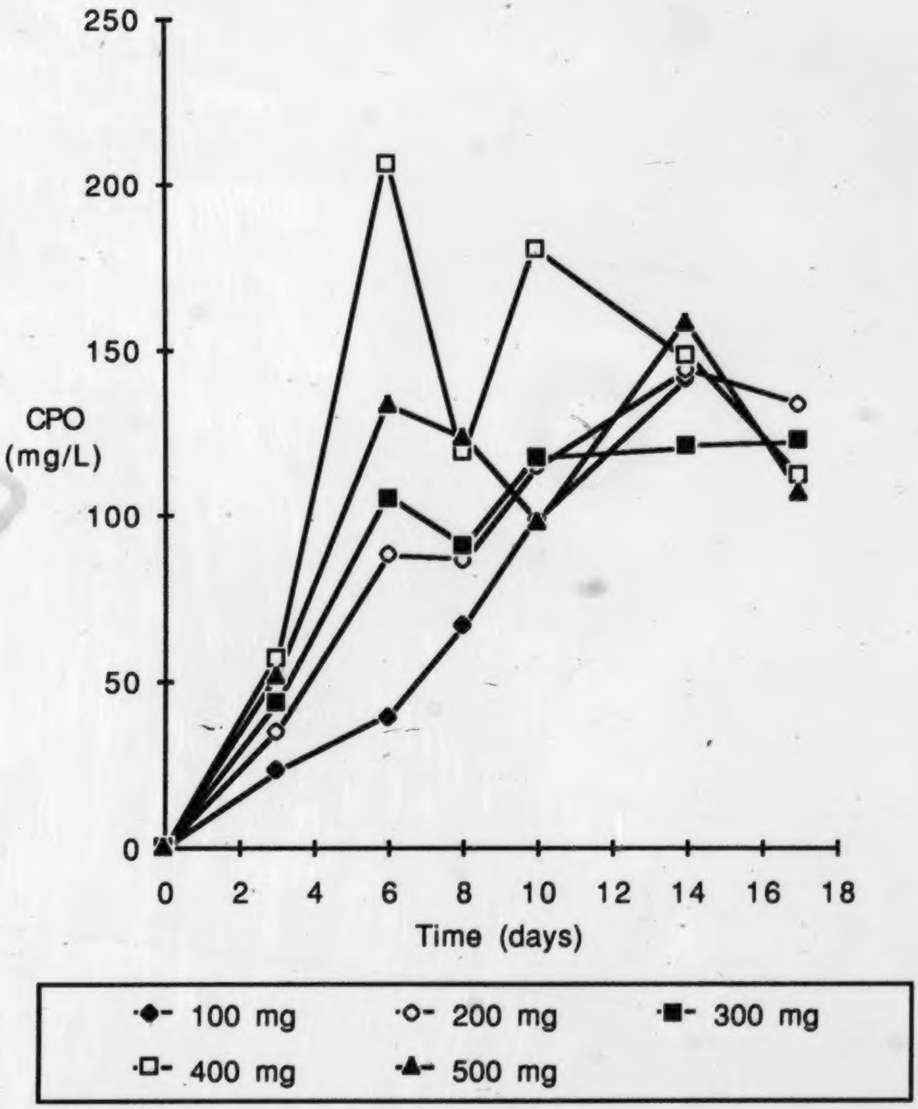
Experiments were carried out to determine the effectiveness of enzyme production by immobilized hyphae in semi-continuous shake flask, batch fermenter and continuously-fed fermenter culture. The results are presented in the following sub sections.

3.1.3.1 Growth and Chloroperoxidase Production by Immobilized *C. fumago*

Once mycelial fragments had been immobilized in κ-carrageenan, the characteristics of CPO production in FS medium were followed and compared to the known CPO production profile of free mycelium (Pickard, 1981). The three strains chosen for immobilization were CMI 89362,

Figure 4. Effect of free hyphae inoculum size on CPO production levels.

C. fumago CMI 89362 was precultured for 11 days in PFS medium, then washed and homogenized in 4% FS. A measured wet weight of mycelium was used to inoculate 200 mL 4% FS in 500 mL Erlenmeyer flasks, as shown in the figure legend. The culture supernatant was assayed periodically for enzyme activity.


250
200
150
100
50
0
CPO
(mg/L)
0
2
4
6
8
10
12
14
16
18
Time (days)
◆ 100 mg
○ 200 mg
■ 300 mg
□ 400 mg
▲ 500 mg

ATCC 11925 and DOAM 137632 based on the reasons stated previously.

The characteristics of CPO production by immobilized *C. fumago* strain CMI 89362 in FS medium in shake flask culture are shown in Fig. 5. Initially the enzyme level was low but it rose to a maximum of 110 mg CPO/L after 10 days. The pH of the medium dropped from an original value of 5.0 to 4.2 and remained at that level for 7 days. While the pH remained stable enzyme production increased, however after 11 days the enzyme level fell due to irreversible denaturation, and was coincident with a pH rise to 6.0. The profiles of pigment production, pH change and fructose utilization for the immobilized cells were found to be very similar to those reported previously for free cells (Pickard, 1981). However, the enzyme levels achieved in immobilized hyphae shake flask cultures were about 70% of levels reached in free hyphal shake flask cultures (120 as opposed to180 mg CPO / L). The increase in bead size diameter over a single transfer was approximately 1 mm and was independent of the initial bead diameter (3 mm). Fructose was not limiting and much remains in the medium after enzyme production has stopped. Pigment production continued throughout the period of the experiment and its formation is believed to involve chloroperoxidase.

3.1.3.2 Effect of Different Washing Procedures

Culture in PFS medium accelerates growth and enzyme production compared to growth in FS medium. However, the carry over of complex nutrient components from the inoculum culture, either in the medium or the cells maybe responsible for pigmentation development or the sloughing of mycelium at the bead surface. To investigate these above possibilities, a variety of washing procedures were evaluated (see Table 2) as a means of depleting the carrageenan immobilized mycelium of nutrients accumulated in the complex medium and to leach out nutrients inside the beads. The washing procedure also served to remove any hyphal fragments sloughed off from the fungal beads.

The CPO levels produced by these cultures were found to vary between 84-111% of the

Figure 5. Culture profile of immobilized hyphae in shake flask.

C. fumago CMI 89362 was immobilized in 4% κ-carrageenan as described in the Methods section. Five hundred mL Erlenmeyer flasks containing 200 mL FS medium were inoculated with 20 mL settled volume of immobilized hyphae. Supernatant samples were taken and analyzed for fructose, CPO, pH, and pigment levels over 15 days.

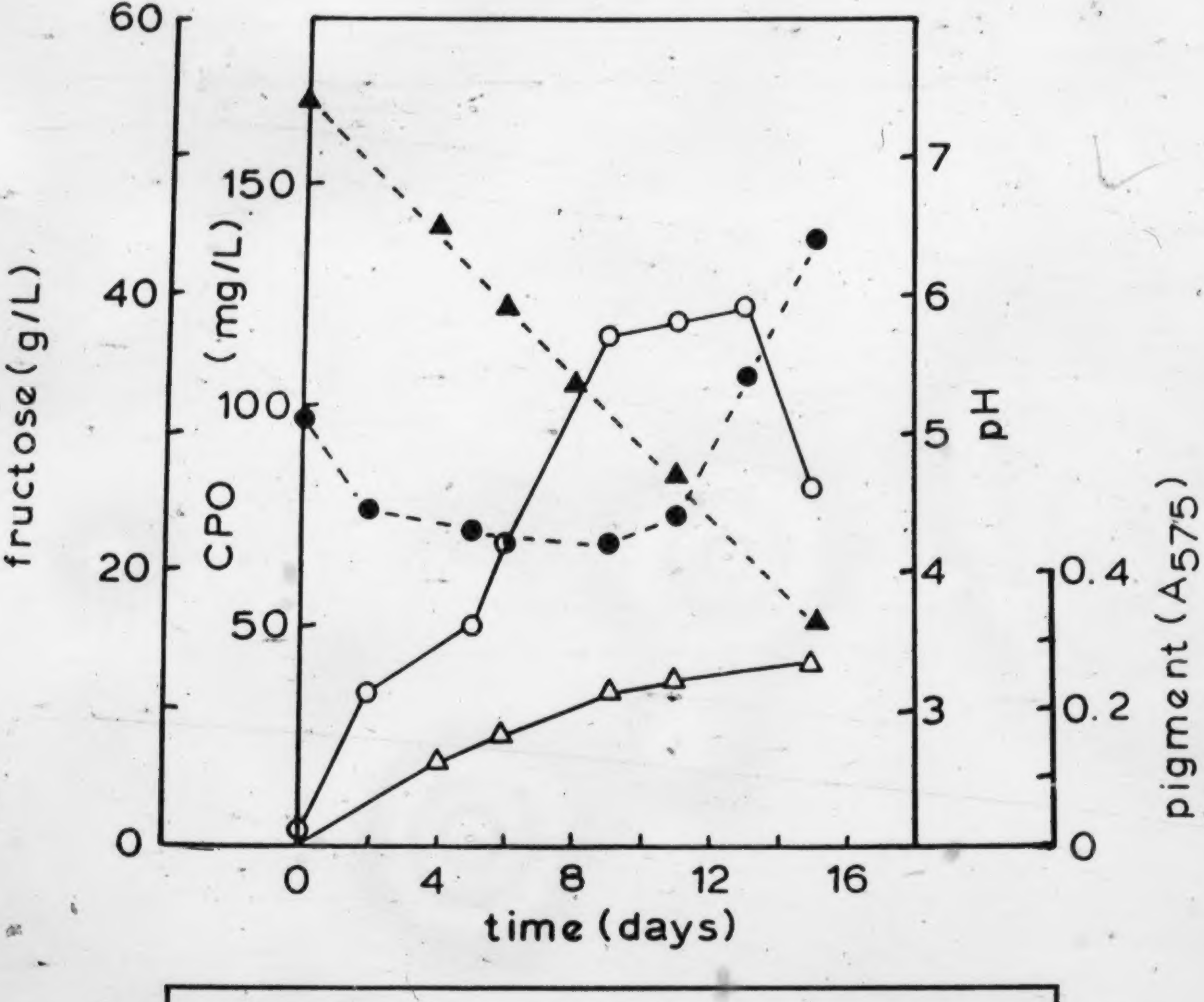

fructose (g/L)
60
40
20
0
CPO (mg/L)
150
100
50
pH
7
6
5
4
3
pigment (A575)
0.4
0.2
0
0
4
8
12
16
time (days)
CPO Level
pH
A 575 nm
Fructose

Table 2. Effect of Different Washing Procedures on Subsequent Enzyme Production.

Type of Wash	CPO Activity (mg/L) Max. Enzyme Conc.	After 16 days
PFS (no wash)	93	74
PFS	90	90
FS	90	90
K Phosphate pH 5.5	100	94
K Phosphate pH 5.5 (2h)	81	64
K Phosphate pH 5.5 (18h)	83	83
10 mM $CaCl_2$	92	92
10 mM $CaCl_2$ (2h)	92	81
10 mM $CaCl_2$ (18h)	100	74

Beads containing immobilized *C. fumago* ATCC 89362 grown in complex medium were suspended in one of four washing media: the complex phytone-fructose salts medium, the defined fructose salts medium, 20 mM potassium phosphate buffer pH 5 and 10 mM calcium chloride. After washing twice in these media by decantation and aspiration, the four groups of beads were divided into three subgroups which were shaken for 0, 2 and 18 h in the wash medium before transfer to fructose salts. The enzyme concentration in the flasks (in duplicate) was followed over the next 16 days.

control values (PFS-no wash), on a random basis, and showed that no treatment was obviously preferable to any other. Also the type of treatment had no long term effect on the culture stability as shown by the high CPO levels after 16 days. Three washes with phosphate buffer were therefore chosen as a standard method of removing free mycelial fragments from cultures between passages.

3.1.3.3 Effect of Immobilized Inoculum Size on CPO Levels in Fermenter Culture

The best size of the immobilized hyphae inoculum was evaluated using 5 different loadings of airlift fermenters and the results are presented in Fig. 6. Larger loadings resulted in the faster production of higher levels of CPO but this also resulted in the production of higher pigment levels (Fig. 7a) and in the case of the highest loading (90 mL) the culture eventually took on characteristics of a fluidized bed. The pH levels seen were nearly identical for all the inoculum sizes until day 15 when the pH in the two highest inoculum fermenters began to rise (Fig. 7b). The setup and appearance of the experiment was photographed near the end of the experiment (Day 25) and can be seen in Fig. 8.

3.1.3.4 Semi-Continuous CPO Production in Shake Flask Culture

In the next study, the immobilized hyphae were used repeatedly to determine if chloroperoxidase could be produced in a semi-continuous manner. If immobilized hypha were capable of prolonged enzyme production and repeated use in serial transfers this would bypass the need to start precultures from stocks. To achieve this, the beads were transferred to fresh medium when the pH began to rise sharply, i.e. after enzyme production had ceased. Thus the immobilized hyphae shake flask study was extended to follow the long-term semi-continuous production of CPO by immobilized mycelium of three strains of *C. fumago* . These results are summarized in Fig 9.

Figure 6. Effect of immobilized hyphae inoculum size on CPO production in fermenter culture.

Freshly immobilized *C. fumago* CMI 89362 were prepared as described in the Methods section and washed with 4% FS. Different settled volumes of the beads were used to inoculate the fermenters; 10 mL, 20 mL, 40 mL, 70 mL, and 90 mL. The beads were dispensed into airlift tower fermenters containing 1L 4% FS and grown as described in the Methods section. Supernatant samples were taken periodically and assayed for CPO activity.

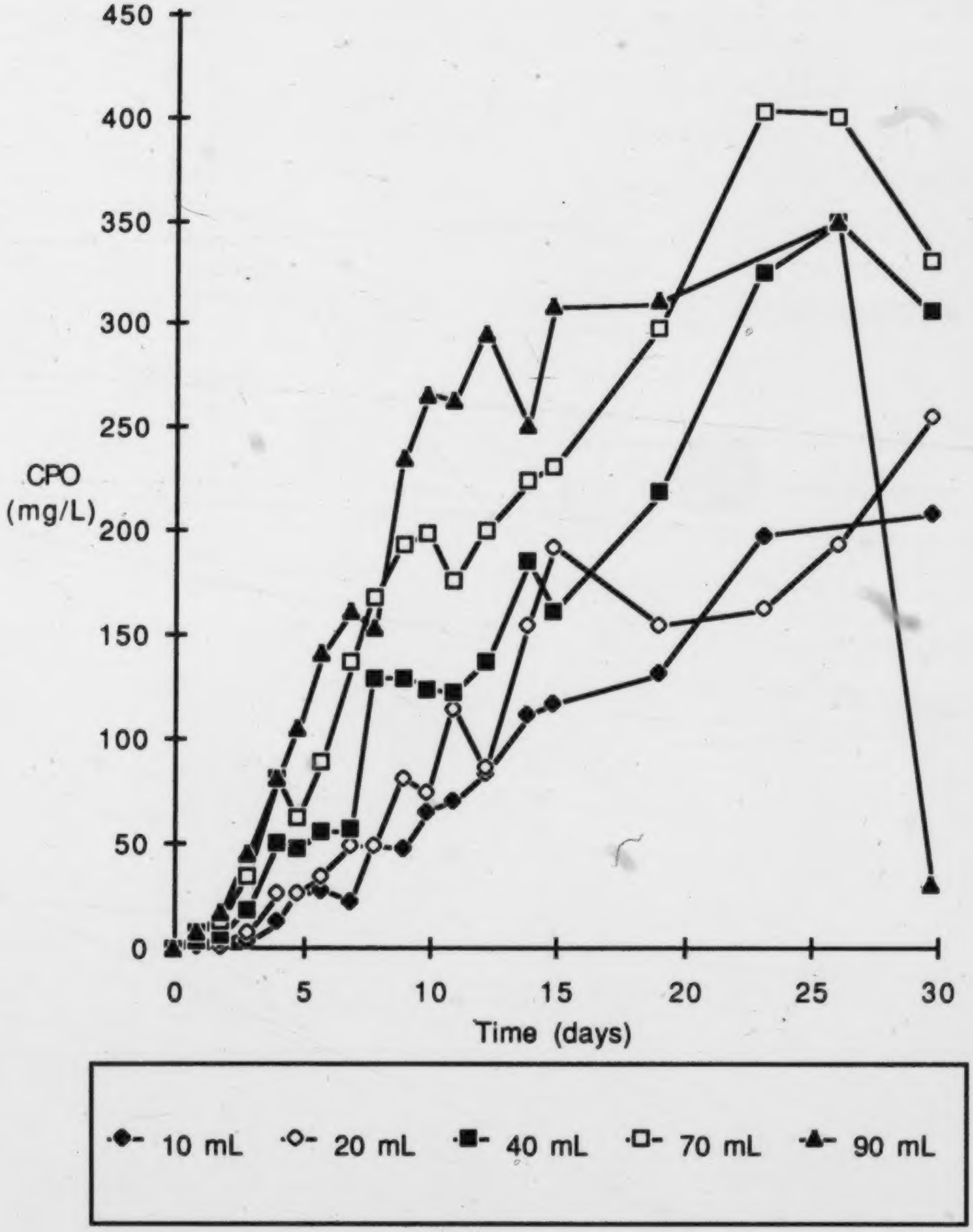
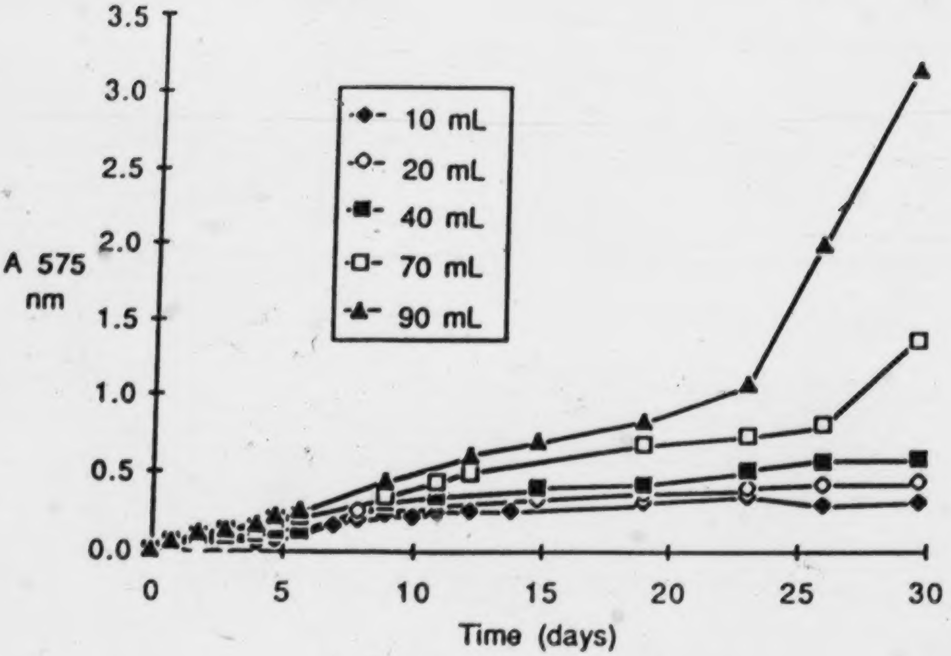

450
400
350
300
250
200
150
100
50
0
CPO
(mg/L)
0
5
10
15
20
25
30
Time (days)
-◆- 10 mL
-◇- 20 mL
-■- 40 mL
-□- 70 mL
-▲- 90 mL

Figure 7. Effect of immobilized hyphae inoculum size on pigment production (a) and pH (b) in fermenter culture.

Freshly immobilized *C. fumago* CMI 89362 were prepared as described in the Methods section and washed with 4% FS. Different settled volumes of the beads were used to inoculate the fermenters; 10 mL, 20 mL, 40 mL, 70 mL, and 90 mL. The beads were dispensed into airlift tower fermenters containing 1L 4% FS and grown as described in the Methods section. Supernatant samples were taken periodically and assayed for pigment and pH levels.


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A 575 nm
10 mL
20 mL
40 mL
70 mL
90 mL
0
5
10
15
20
25
30
Time (days)

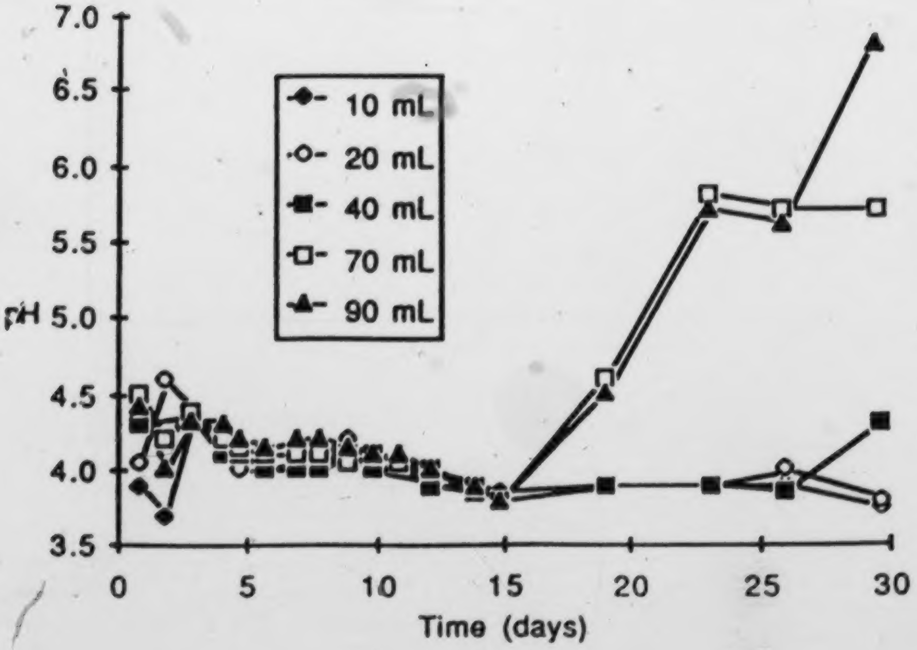
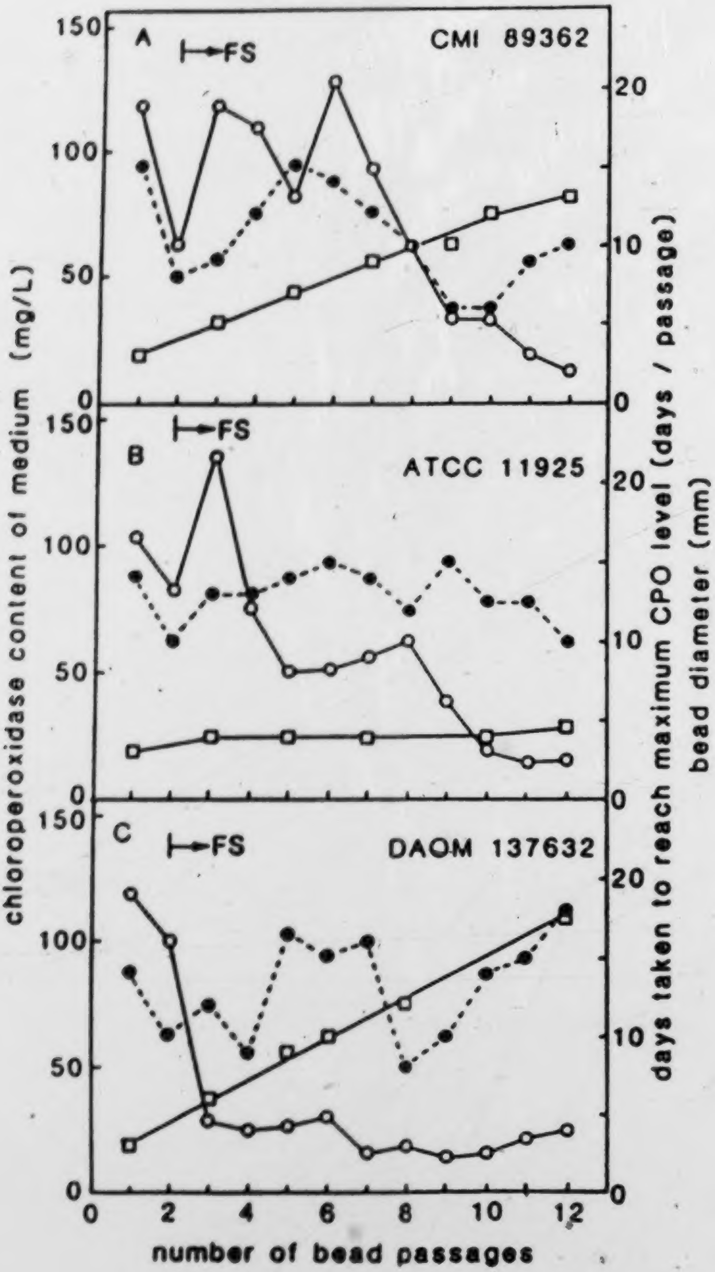

7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
pH
10 mL
20 mL
40 mL
70 mL
90 mL
0
5
10
15
20
25
30
Time (days)

Figure 8. Appearance of immobilized hyphae inoculum size experiment after 25 days.

Freshly immobilized *C. fumago* CMI 89362 were prepared as described in the Methods section and washed with 4% FS. Different settled volumes of the beads were used to inoculate 5 airlift fermenters; 10 mL, 20 mL, 40 mL, 70 mL, and 90 mL, from right to left. The beads were dispensed into airlift tower fermenters containing 1L 4% FS and grown as described in the Methods section and the photograph was taken on day 26.

Figure 9. Semi-continuous production of CPO by immobilized *C. fumago* in shake flask culture.

C. fumago strains CMI 89362, ATCC 11925 and DOAM 137632 were immobilized and washed between transfers with 20 mM phoshate buffer pH 5.5 as described in the Methods section. After immobilization, 20 mL settled volume of beads were used to inoculate 200 mL of PFS medium. After 2 passages in PFS, the beads were transferred to 200 mL 4% FS medium for 10 passages. Supernatant samples were obtained and assayed for enzyme activity. The effect of repeated transfers of immobilized *C. fumago* strains CMI 89362, ATCC 11925 and DOAM 137632 was determined for the maximum enzyme levels (○), the length of time for each passage in days (y_2-axis) (●), and the increase in bead size (□).


A
FS
CMI 89362
B
FS
ATCC 11925
C
FS
DAOM 137632
chloroperoxidase content of medium (mg/L)
days taken to reach maximum CPO level (days / passage)
bead diameter (mm)
number of bead passages
0
50
100
150
0
10
20
0
2
4
6
8
10
12

The initial two passages were in PFS but thereafter in FS medium. In place of a direct time scale on the ordinate is the number of passages, the length of which were not constant but varied depending on the length of time taken to reach maximum enzyme levels before the medium pH rose above 6. This parameter, recorded on the left abscissa, ranged from 8-18 days with a mean of 12 days over the first 8 passages.

The three strains tested behaved differently. With strain CMI 89362, the activity remained high for approximately 96 days or 8 successive transfers in defined medium after initial growth in complex medium. The levels were lower than those attained when free cell batch cultures were used but CMI 89362 was able to produce enzyme concentrations with a mean of 96 mg CPO/L during six production periods. There was variability in the length of time to reach a maximum enzyme level and the increase in bead diameter was almost linear. CPO production by strain ATCC 11925 declined with increasing passage number but appeared to be less variable in the amount of time to reach maximum enzyme levels and bead size remained very constant. The bead size did not increase because little growth occurred on the bead surface. New growth came in the form of fine grains which arose from shed hyphal fragments. Strain DOAM 137632 showed an immediate reduction in its production levels after transfer to defined medium on passage 3 where it remained for the rest of the experiment, yet the culture continued to grow indicated by the bead diameter increase. In summary, on transfer from complex to defined medium there was little change in enzyme production by *C. fumago* CMI 89362 over the first 8 passages (mean : 96 mg CPO/L). In strain ATCC 11925 enzyme production declined gradually over this period (mean: 69 mg CPO/L) while in the third culture, DOAM 137632, production fell suddenly (mean: 23 mg CPO/L).

The time at which the supernatant was harvested was important as enzyme activity is lost when the medium pH rises above 6.5. However exposure of the immobilized cultures to these higher pH values did not affect subsequent enzyme production in later passages, indicating that the stability of the enzyme producing system was not affected.

During each passage, the fresh fructose salts medium stimulated new mycelial growth on the bead surface. Mycelial fragments were sloughed off into the medium and these were removed during the washing procedure. The surface growth also caused an increase in bead size from an initial diameter of 0.3 cm to up to 1 cm. Two of the cultures, CMI 89362 and DOAM 137632, showed an almost linear increase in bead dimension while the intermediate producer ATCC 11925 showed virtually none. This increase became a problem when beads reached 1 cm diameter as they were then in constant contact with each other, causing physical breakdown, probable decreased oxygen transfer and eventually lower enzyme production levels.

No problems were encountered with bacterial or yeast contamination even though the beads were extensively manipulated over the 5 months of the experiment.

3.1.3.5 Continuous Production by Immobilized Hyphae

Immobilized *C. fumago* CMI 89362 was used for continuous production of chloroperoxidase in an external loop airlift tower fermenter in defined medium. The fungal beads were circulated until the enzyme level in the medium reached >100 mg/L before medium replacement began. The effluent from the column was sampled over 19 days and a plot of the enzyme content against time is seen in Fig. 10. The chloroperoxidase level followed the theoretical washout curve for the first 2 days but then maintained a level of 40-50 mg CPO/L.

Depending on the fermenter design, the rheology of the medium displayed during operation of a fermenter may show characteristics of a complete mix fermenter system. In a complete mix system the concentration of an inert compound at any time in the fermenter follows a mathematically derived equation for the compound's washout, assuming it is not being continuously produced or transformed. In Fig. 11, the complete mix characteristics of the airlift fermenter is illustrated by the washout of methylene blue from a methylene blue/distilled water solution by continuous distilled water addition.

Figure 10. Continuous CPO production by repeatedly used immobilized hyphae.

Immobilized *C. fumago* CMI 89362 was incubated in PFS and 4% FS medium before the inoculation of 50mL settled volume into a fermenter containing 1L 4% FS medium. Fermenter parameters were: room temperature, 0.6 L air/min, bead circulation time= 5 s, pump flow rate=23 mL/h (HRT=43 h). The fermenter was operated as a batch system (conditioning phase) for 9 days to allow CPO levels to rise above 100 mg/L prior to medium addition. Effluent from the fermenter was assayed for CPO activity and compared to a theoretical washout curve*.

* see Fig. 9 for washout out curve calculation.

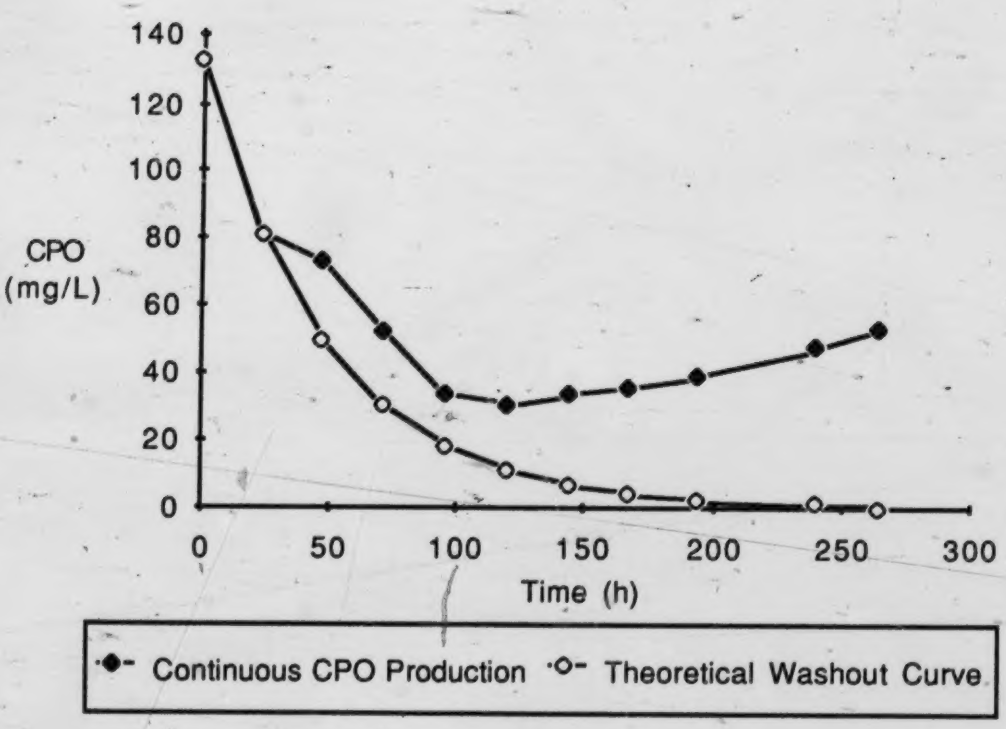
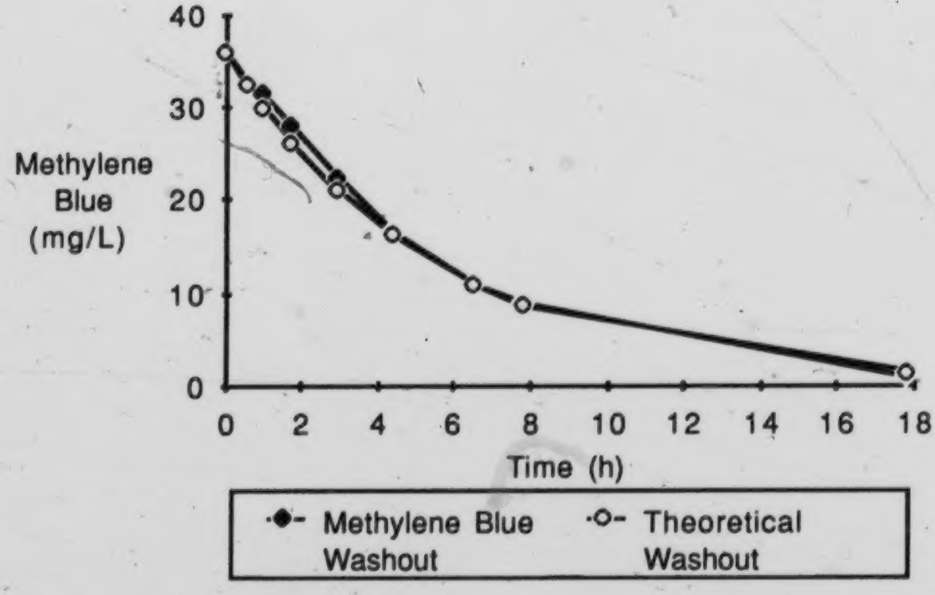

CPO (mg/L)
140
120
100
80
60
40
20
0
0
50
100
150
200
250
300
Time (h)
◆ Continuous CPO Production
○ Theoretical Washout Curve

Figure 11. Fermenter design analysis. Determination of a complete mix system.

Methylene blue was washed out of the fermenter by simultaneous distilled water addition and effluent removal. The flow rate was 180 mL/h (HRT=5.5 h) and the aeration rate was 0.6 L air/min. The concentration of methylene blue in the fermenter effluent was followed by spectrophotometric assay (λmax=668 nm). The initial concentration of methylene blue (M.B.) in the fermenter was then used to calculate a theoretical washout curve using the formula for a complete mix system: $C_t = C_o e^{-t/t_o}$, where C_t = the concentration of M.B. at time t, C_o = the concentration of M.B. at time = 0, t = time, and t_o = hydraulic retention time (HRT = system volume/flow rate).


40
30
20
10
0
Methylene Blue (mg/L)
0
2
4
6
8
10
12
14
16
18
Time (h)
Methylene Blue Washout
Theoretical Washout

3.1.3.6 Comparison of Immobilized Mycelium with Free Mycelial Pellets

The results obtained with immobilized hyphae were encouraging but the number of manipulations and the time taken to prepare the inocula for each fermenter run was a major disadvantage to using immobilized hyphae. For this reason, comparisons were made with mycelial pellets in airlift fermentations. The pellets were generated by manipulation of conditions for inoculum preparation mentioned earlier.

Pellet formation directed by immobilizing homogenized mycelia in κ-carrageenan and by controlled inoculum preparation were compared in Fig. 12. Using a different inoculum but under the same growth conditions the free pellets showed the similar levels of continuous CPO production (30-50 mg CPO/L) as the κ-carrageenan beads. These immobilized mycelium results are those reported in Fig. 10.

3.1.4 Continuous Production by Mycelial Pellets

A number of fermenter cultures were investigated using mycelial pellets instead of immobilized hyphae to determine the effect of varying experimental parameters. *C. fumago* CMI 89362 pellets were grown in 4% FS medium in an airlift fermenter, as described for the immobilized hyphae, and were monitored for medium levels of enzyme production, carbohydrate and nitrate, pH, and pigment production.

The biomass level in the fermenter could not be determined at any particular time during an experiment but the inoculum size, the pellet size increase and the final biomass yield from the fermenter were determined for each experiment. A culture profile of a typical continuous CPO production fermenter run is shown in Fig. 13. Enzyme levels followed the washout curve for the first 2 retention times, when enzyme levels were allowed to rise initially above 100 mg/L (conditioning). The culture produced CPO at 30-50 mg/L for >4 retention times. After conditioning, the pigment levels were high and the sugar and nitrate levels were low. Over time, total carbohydrate and nitrate levels rose almost to the levels in the feed, while pigment

Figure 12. Comparison of continuous production by immobilized hyphae and mycelial pellets.

The fermenter was inoculated with a measured settled volume of mycelial pellets of *C. fumago* CMI 89362 grown in a shake flask. The pellets were circulated (pellet circulation time = 5 s, aeration rate = 0.6 L/min) without addition or removal of medium until the medium CPO level had reached an elevated level (fermenter conditioning), above 60 mg CPO/L. Then simultaneous medium (4% FS) addition and removal began (HRT= 43 h).The effluent was sampled for at least 4 retention times.

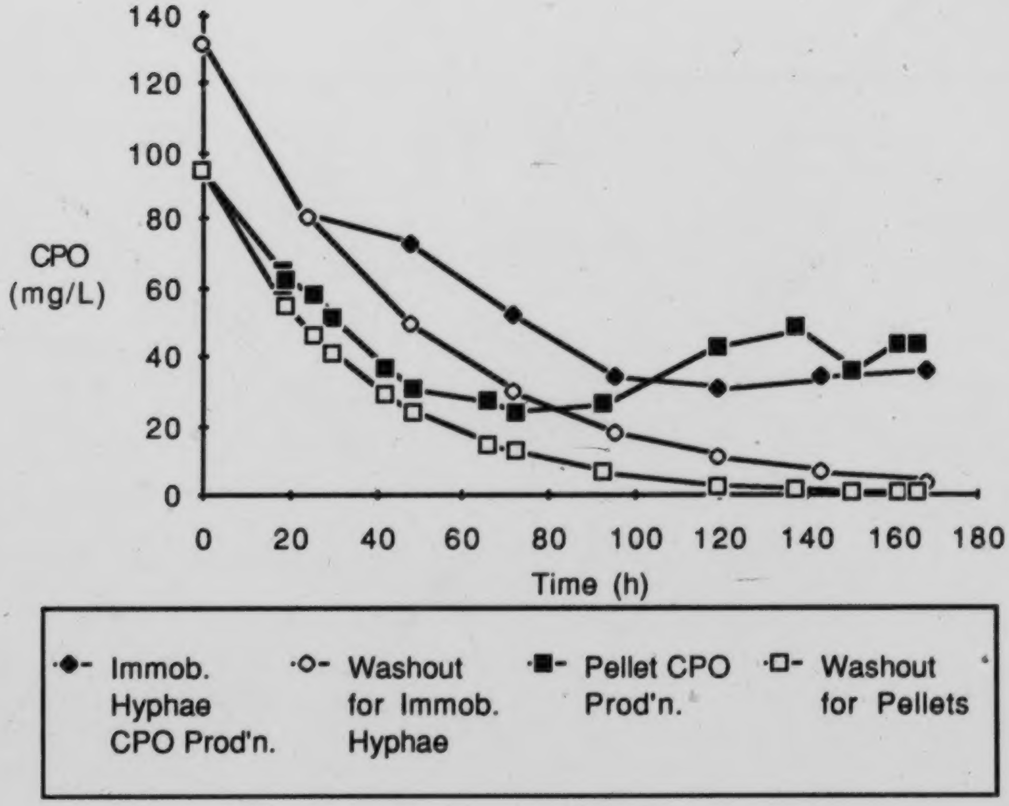
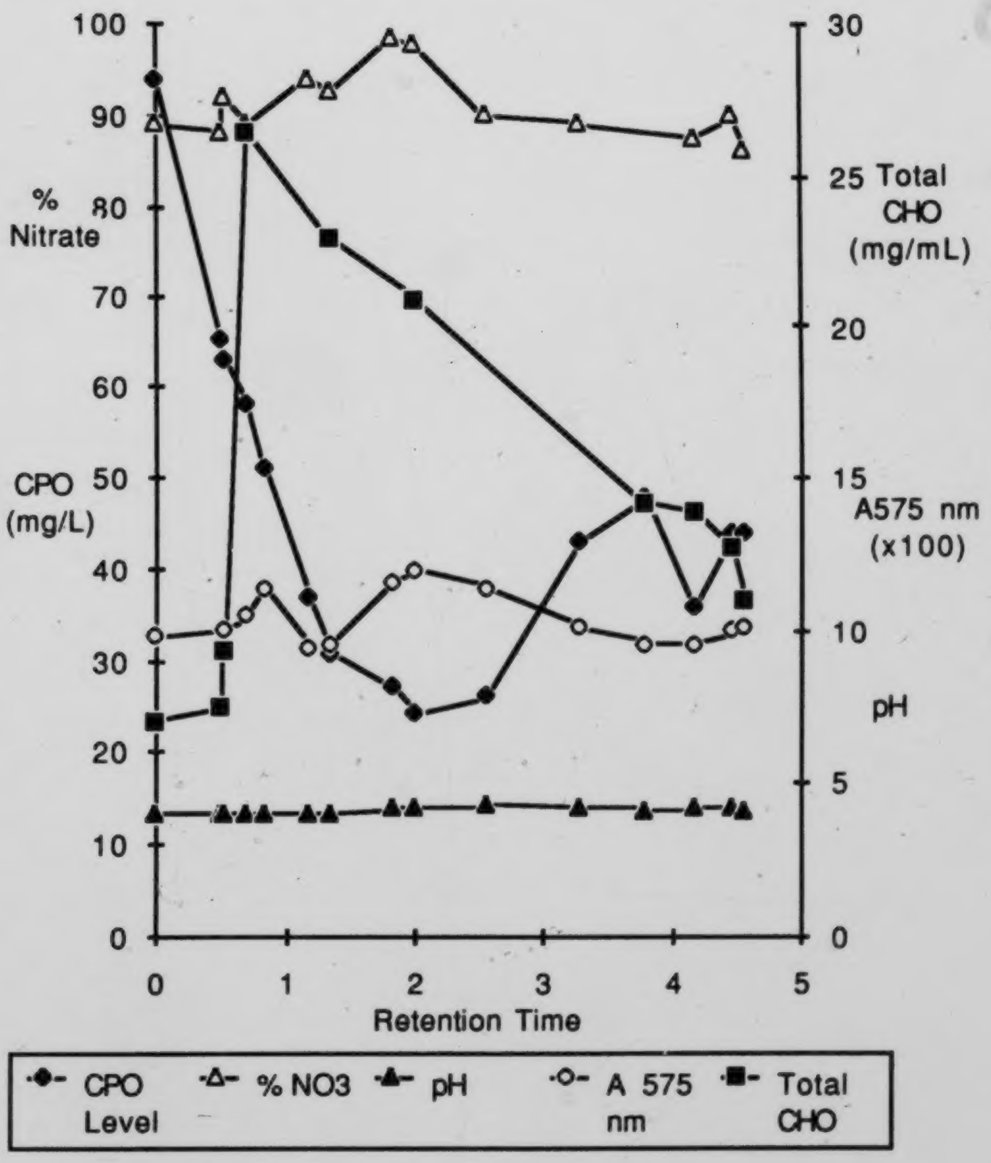

140
120
100
80
60
40
20
0
CPO (mg/L)
0
20
40
60
80
100
120
140
160
180
Time (h)
-◆- Immob. Hyphae CPO Prod'n.
-○- Washout for Immob. Hyphae
-■- Pellet CPO Prod'n.
-□- Washout for Pellets

Figure 13. Fermenter culture profile of mycelial pellets continuously-fed with 4% FS.

A 1 L fermenter was inoculated with 50 mL settled volume of *C. fumago* CMI 89362 mycelial pellets grown in a shake flask. The pellets were circulated once every 5 s with an aeration rate of 0.6 L/min without addition or removal of medium until the medium CPO level had reached an elevated level (fermenter conditioning), above 90 mg CPO/L. Then simultaneous medium addition of 4% FS and removal of culture fluid began at a flow rate of 28 mL/h (HRT=36 h). The effluent was sampled for 4 retention times and assayed for CPO, nitrate, pH, pigment and total carbohydrate (CHO) levels.


100
90
80
70
60
50
40
30
20
10
0
%
Nitrate
CPO
(mg/L)
30
25 Total
CHO
(mg/mL)
20
15
A575 nm
(x100)
10
pH
5
0
0
1
2
3
4
5
Retention Time
-●- CPO Level
-△- % NO3
-▲- pH
-○- A 575 nm
-■- Total CHO

levels remained fairly constant throughout. However pigment levels did decrease to 1/3 of the starting level in other fermenter cultures at flow rates above 65 mL/h (data not shown). The pH of the effluent fluctuated between 3.5 and 4.5. Pellet diameter size increased linearly over time, from less than 3 mm diameter to approximately 10 mm and the final biomass levels were found to be 15-20 g dry weight/L from an initial inoculum of 0.1 g dry weight/L.

The effect of nutrient feed (4% FS) rate on the levels of CPO found in the effluent is shown in Fig.14. Several flow rates were tested; slow (30mL/h), moderate (40-60 mL/h), and fast (120 mL/h). Increasing the flow rate of the nutrient feed from moderate to fast succeeded only in reducing the levels of CPO found in the effluent from 30-50 mg CPO/L to < 10 mg CPO /L. Enzyme washout was almost complete at the high flow rate whereas at low flow rates (15 mL/h) the enzyme levels in the effluent were very close to control run batch levels.

The carbohydrate levels in the effluent were still high when using a 4% FS feed. In order to observe the effect of lowered fructose concentration in the nutrient feed on CPO levels found in the effluent, experiments were conducted using a 2% FS feed solution, since half the fructose remained in the batch cultures. The profiles of enzyme concentration found in the effluent using 2% FS at three different flow rates (Fig.15) showed that 2% FS feed solution was capable of supporting continuous enzyme production. However, there was a slight lowering in CPO production levels when compared with 4% fructose-salts medium (Fig 14). Variation in the CPO levels could be explained by the slight differences in the flow rates at each level and inoculum variation. Thus a 2% FS feed seemed to be adequate for the maintenance of the culture during continuous enzyme production. The mycelial pellets used repeatedly in these continuous-fed fermenter cultures can be viewed in Fig. 16.

It was also determined that the starting time of nutrient feed addition had no effect on the final level of CPO produced (Fig.17 and 18). When simultaneous addition and removal of medium (4% FS-slow flow rate) was initiated at the time of inoculation, the enzyme

Figure 14. Effect of 4% FS feeds on effluent CPO levels from airlift fermenter culture.

C. fumago CMI 89362 pellets were used for all three experiments. Fermenter culture conditions were the same for all three experiments and were the same as those described in the methods section. CPO levels were monitored in the culture effluent and plotted against the retention time.

Slow Flow Rate

Fungal pellets were generated as described in the methods section. In the first experiment 60 mg of pellets (0.1cm diameter, 10 mL settled volume) were inoculated into a fermenter containing 1L 4% FS medium. After conditioning, 4% FS medium was added and culture supernatant was removed at a slow flow rate (28 mL/h, HRT=36h). The same pellets were reused in the next experiment.

Moderate Flow Rate

The pellets (1.2 cm diameter, 100 mL settled volume, 5 g dry wt.) obtained from the slow feed experiment were washed with 0.1 M phosphate buffer pH 5.5 and returned to a sterilized fermenter containing fresh 4% FS medium. After conditioning simultaneous medium addition and supernatant removal was carried out a moderate flow rate (46 mL/h, HRT=22h).

Fast Flow Rate

For this experiment fresh pellets were produced as previously described. The fermenter was inoculated with a measured settled volume (30 mL) of pellets (0.5 cm diameter, 1.2 g dry wt.). Again after conditioning, the 4% FS medium feed and supernatant sampling took place at a fast flow rate (114 mL/h, HRT=8.8h).

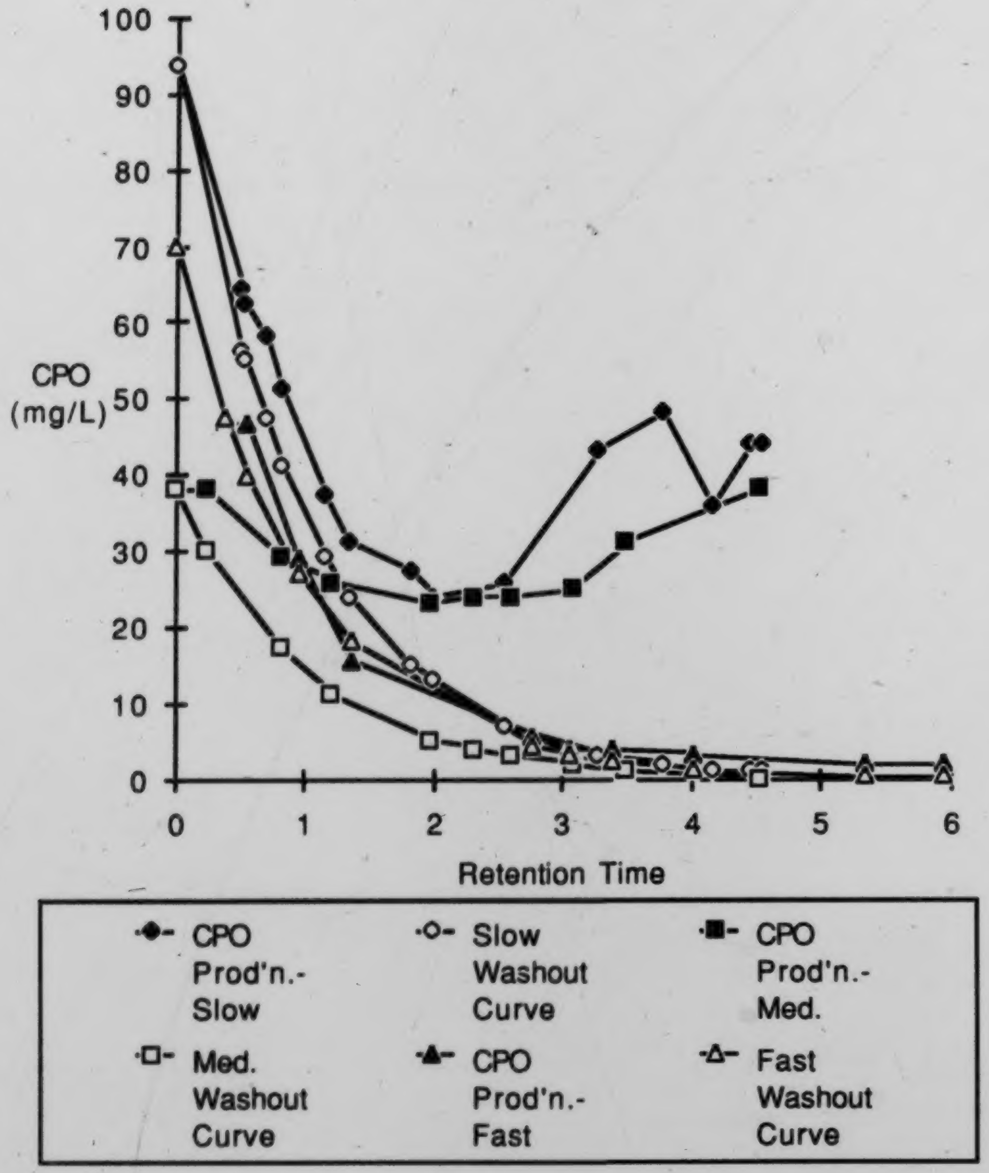

CPO (mg/L)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Retention Time
-◆- CPO Prod'n.-Slow
-○- Slow Washout Curve
-■- CPO Prod'n.-Med.
-□- Med. Washout Curve
-▲- CPO Prod'n.-Fast
-△- Fast Washout Curve

Figure 15. Effect of 2% FS feeds on effluent CPO levels from airlift fermenter culture.

Fermenter culture conditions were the same for all three experiments, as described in the methods section. *C. fumago* CMI 89362 pellets were used for all three experiments.

Fast Flow Rate

In this experiment previously used pellets (1.3 cm diameter, 250 mL settled volume, 27 g dry wt.) were washed with 0.1 M phosphate buffer and inoculated into a fermenter containing 1L 2% FS medium. After conditioning, 2% FS medium was added and culture supernatant was removed at a fast flow rate (120 mL/h, HRT=8.6 h). The same pellets were reused in the next experiment.

Moderate Flow Rate

The pellets (1.4 cm diameter, 350 mL settled volume, 30 g dry wt.) obtained from the above fast feed experiment were washed with 0.1 M phosphate buffer pH 5.5 and returned to a cleaned and resterilized fermenter containing fresh 2% FS medium. After conditioning, simultaneous medium addition (2% FS) and supernatant removal was carried out a moderate flow rate (65 mL/h, HRT=15.3 h).

Slow Flow Rate

For this experiment pellets were saved from the previous fermenter run. The fermenter containing 2% FS was inoculated with a measured settled volume (300 mL) of pellets (1.6 cm diameter, 30 g dry wt.). Again after conditioning, the 2% FS medium feed and supernatant sampling took place at a slow flow rate (33 mL/h, HRT=30.4 h).

CPO levels were monitored in the culture effluent and plotted against the retention time.

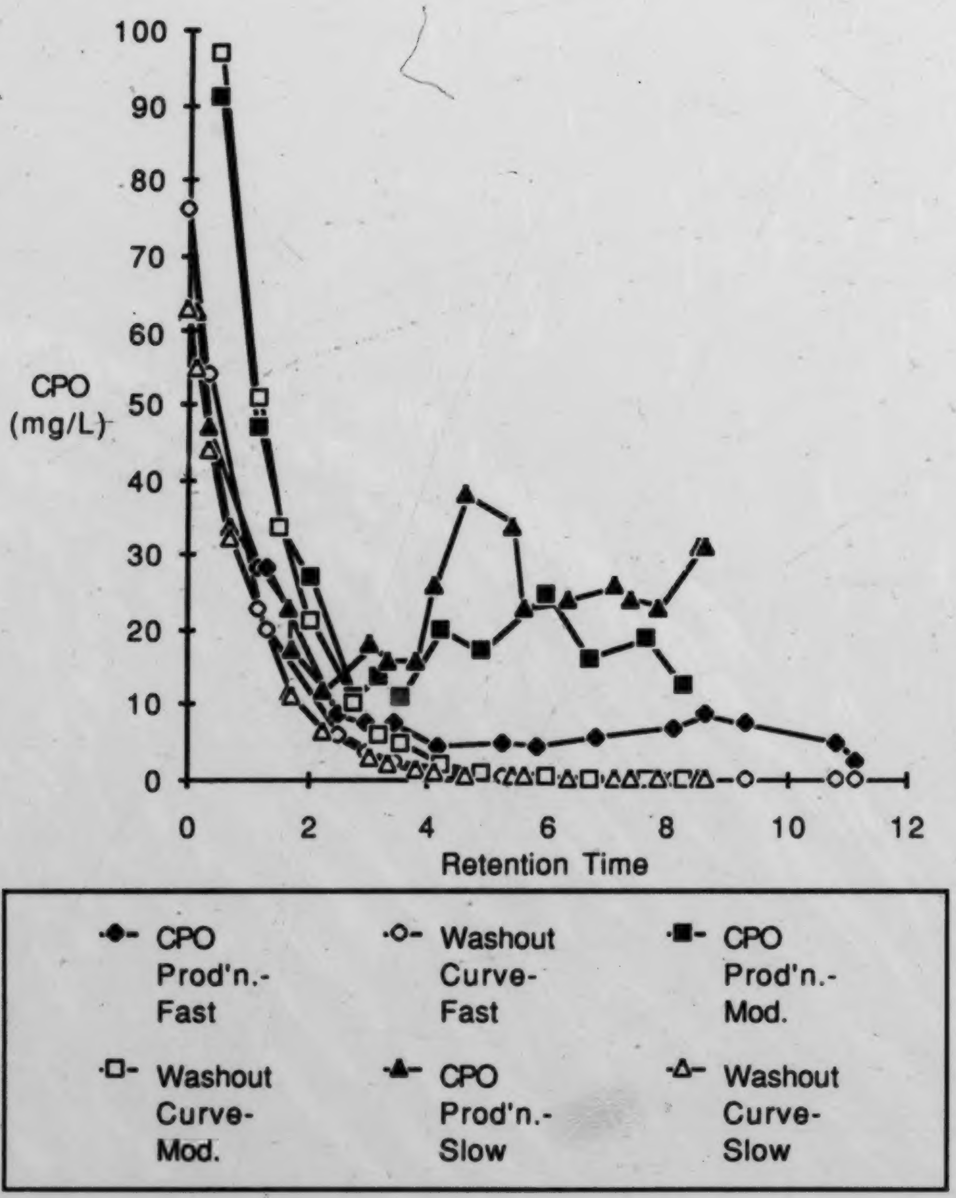

100
90
80
70
60
50
40
30
20
10
0
CPO
(mg/L)
0
2
4
6
8
10
12
Retention Time
-◆- CPO Prod'n.- Fast
-○- Washout Curve- Fast
-■- CPO Prod'n.- Mod.
-□- Washout Curve- Mod.
-▲- CPO Prod'n.- Slow
-△- Washout Curve- Slow

Figure 16. Photograph of mycelial pellets used for continuous CPO production.

After 4 fermenter cultures, the mycelial pellets were firm and spherical in shape with a highly convoluted surface. The black pellets ranged in size from 1.3-1.5 cm in diameter.

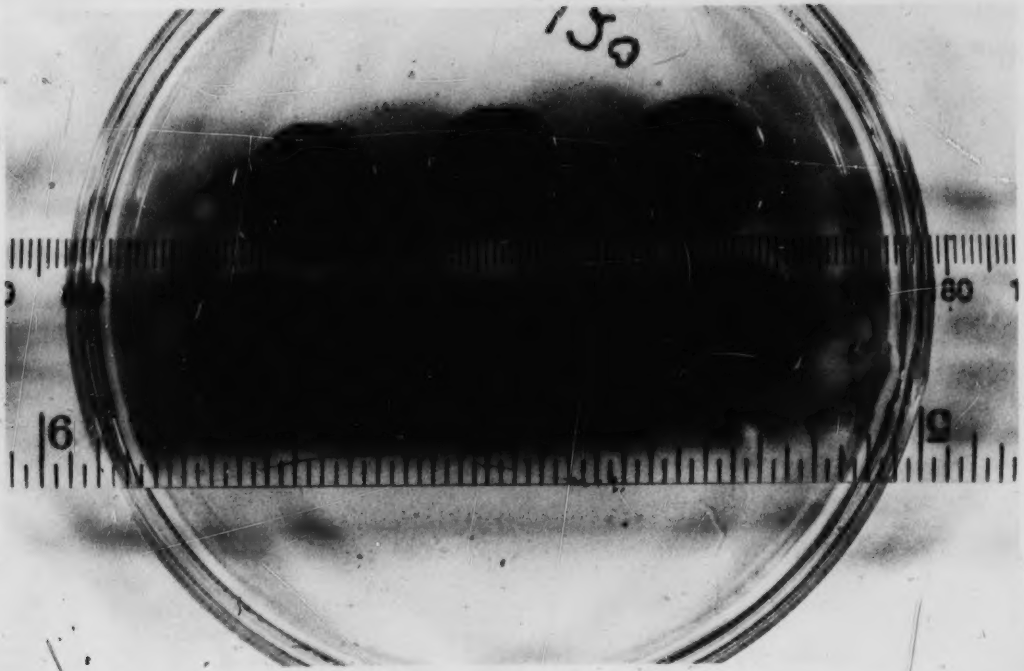
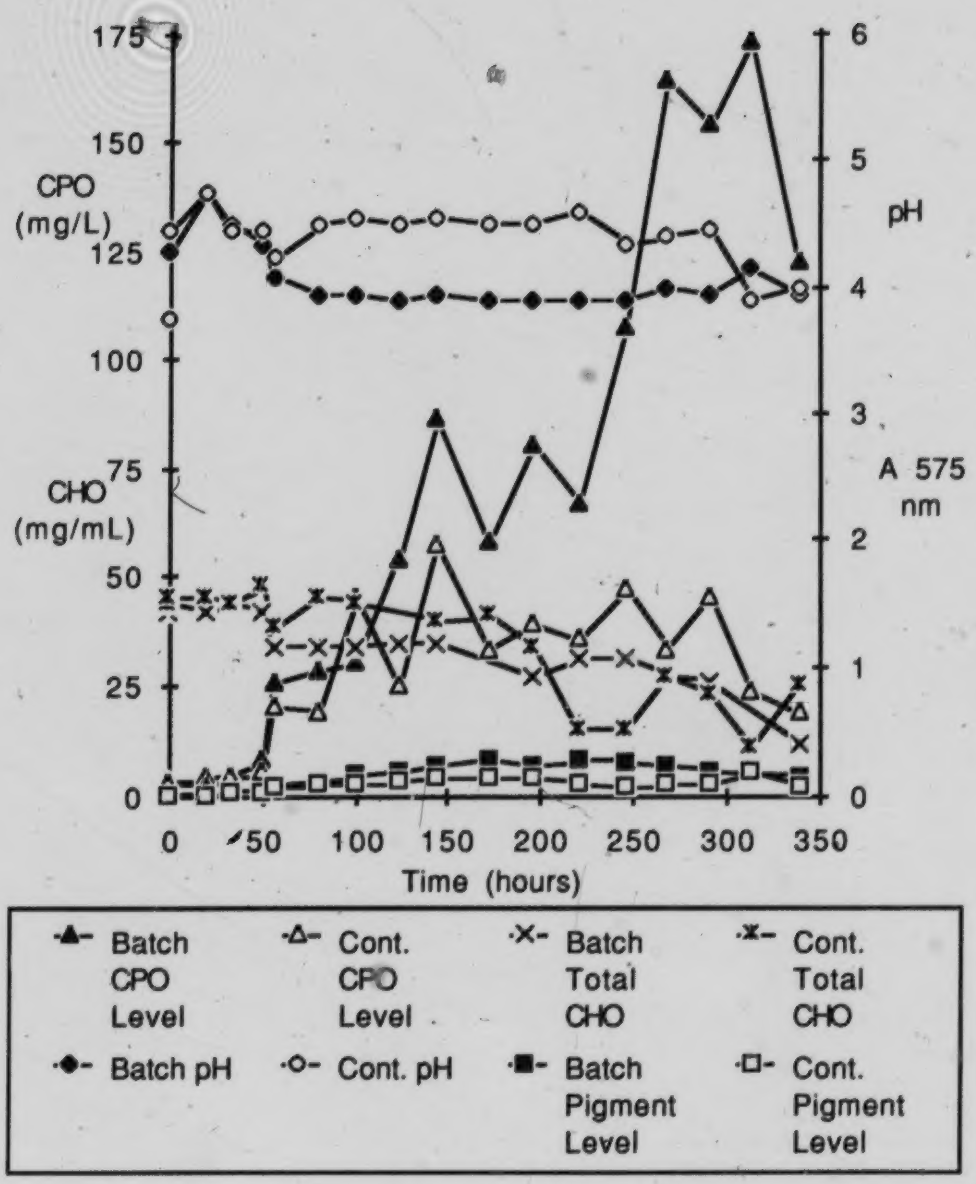

13a
6
80
9

Figure 17. Fermenter culture profile and effect of culture feed time on subsequent CPO production levels.

A 50 mL settled volume (2.5 g dry wt.) of *C. fumago* CMI 89362 fungal pellets were transferred to each of two airlift fermenters, each containing 1 L 4% FS medium. At inoculation time, one fermenter was given a 4% FS feed at a slow flow rate (26 mL/h, HRT= 38.5 h) while the other fermenter was operated as a batch culture. CPO, carbohydrate (CHO), pigment and pH levels were monitored from both fermenters and were plotted as a function of the time.


CPO (mg/L)
CHO (mg/mL)
pH
A 575 nm
175
150
125
100
75
50
25
0
6
5
4
3
2
1
0
0
50
100
150
200
250
300
350
Time (hours)
-▲- Batch CPO Level
-△- Cont. CPO Level
-X- Batch Total CHO
-✱- Cont. Total CHO
-◆- Batch pH
-○- Cont. pH
-■- Batch Pigment Level
-□- Cont. Pigment Level

concentration climbed and eventually stabilized at 30-50 mg CPO/L (Fig. 17). This result is very similar to the conditioning experiments where the enzyme levels were first allowed to climb above 100 mg CPO/L before simultaneous medium addition and removal began (see Fig. 14). Figure 17 also shows that the pH and the pigment levels remain very constant and the total carbohydrate levels never fall below 10 mg/mL in the batch or the continuously fed fermenter. At a slow flow rate (fermenter conditioning experiment not done) enzyme levels could be elevated to approximately 100 mg CPO/L, and closely followed the batch CPO production profile (Fig. 18).

In a composite graph containing the pooled data from all continuous CPO production experiments, Fig. 19 relates the average level of enzyme produced in continuously fed cultures to the flow rate of the nutrient feed. Slow flow rates (<25 mL/h) were found to be the best for high levels of continuous enzyme production. At flow rates below approximately 65 mL/h, as shown in Fig. 20, the rate of enzyme production in the continuously fed culture was 1.19±.09 mg CPO/h. At flow rates above 65 mL/h, the rate of production dropped.

The use of PFS medium as a growth medium was investigated (data not presented). It was discontinued when the immobilized hypha grew in 10 days to a size >2 cm, and eventually plugged the downcomer tube of the fermenter (Fig. 21). During growth the fermenter culture foamed excessively and the aeration rate had to be lowered. Using the same size of inoculum in 2% FS and 4% FS (data not shown) resulted in a more manageable yield of fungal biomass. When 2% fructose-salts was used to support fungal growth it resulted in slightly lower enzyme levels (Pickard, 1981), so 4% FS was used for enzyme production. Also when using the defined medium, little (0.01%-0.1%) or no antifoam was required.

Biomass determinations were attempted, harvesting whole fermenters to obtain single value. This relationship is presented in Fig. 22. Fungal dry weight was correlated to the settled pellet volume and the pellet diameter. For the given inoculum size, the dry weight was directly proportional to the settled volume of pellets in the fermenter (data not shown) but both the dry

Figure 18. Comparison of enzyme production under batch and slow-feed rate continuous conditions in airlift fermenters.

Freshly prepared *C. fumago* CMI 89362 pellets (see Methods section) were inoculated (10 mL settled vol/fermenter, 0.37 g dry wt.) into each of two airlift fermenters containing 4% FS medium. At inoculation time one fermenter began to receive a 4% FS feed at a very slow flow rate (15 mL/h, HRT= 66 h) while the other fermenter was operated as a batch culture. CPO and pH levels were monitored from both fermenters and were plotted as a function of the time.

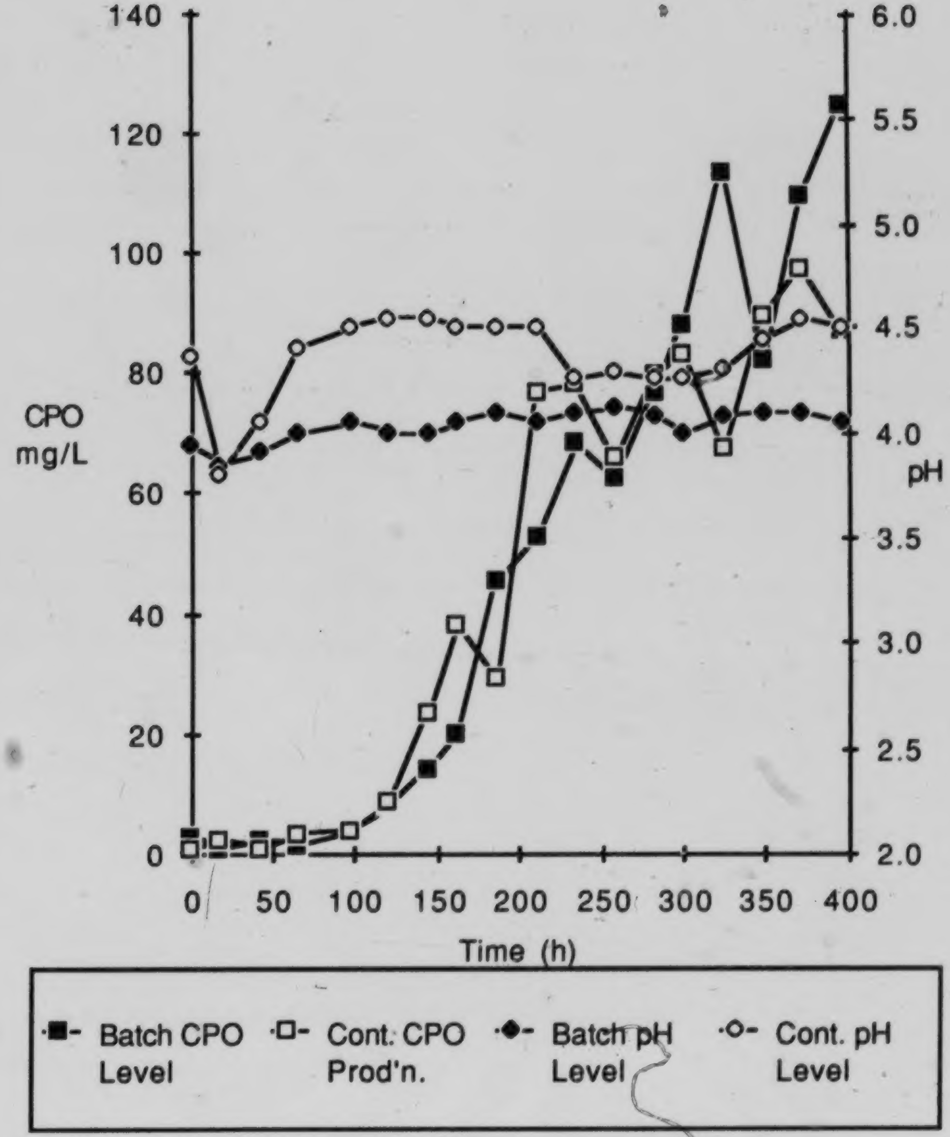

140
120
100
80
60
40
20
0
CPO mg/L
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
pH
0
50
100
150
200
250
300
350
400
Time (h)
■ Batch CPO Level
□ Cont. CPO Prod'n.
◆ Batch pH Level
○ Cont. pH Level

Figure 19. Effect of flow rate on continuous CPO production from airlift fermenter cultures.

The data from the previous continuously fed fermenter cultures are summarized and presented in this graph. The mg CPO/L value was calculated by averaging the CPO levels found in the effluent after 3 retention times.

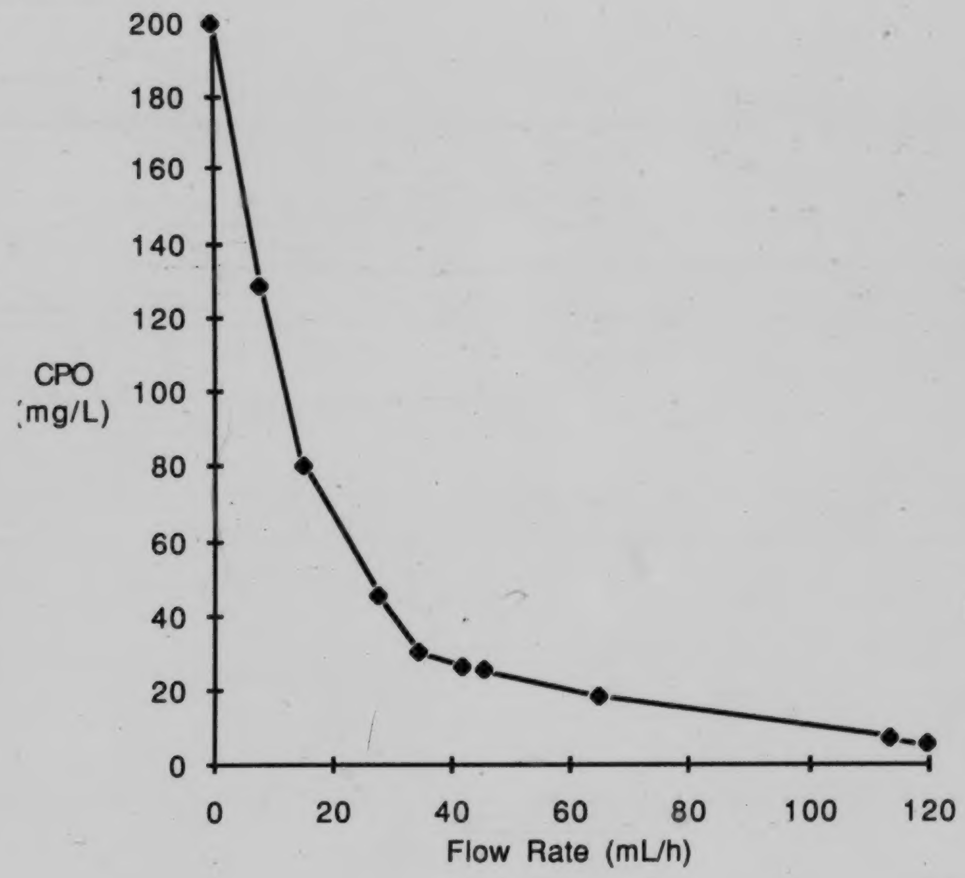

200
180
160
140
120
100
80
60
40
20
0
CPO
(mg/L)
0
20
40
60
80
100
120
Flow Rate (mL/h)

Figure 20. Effect of flow rate on continuous CPO production from airlift fermenter cultures.

The rate of CPO production in mg per hour as a function of the flow rate of the feed was plotted. The production rate was determined by dividing the average CPO level after 3 retention times by the flow rate.

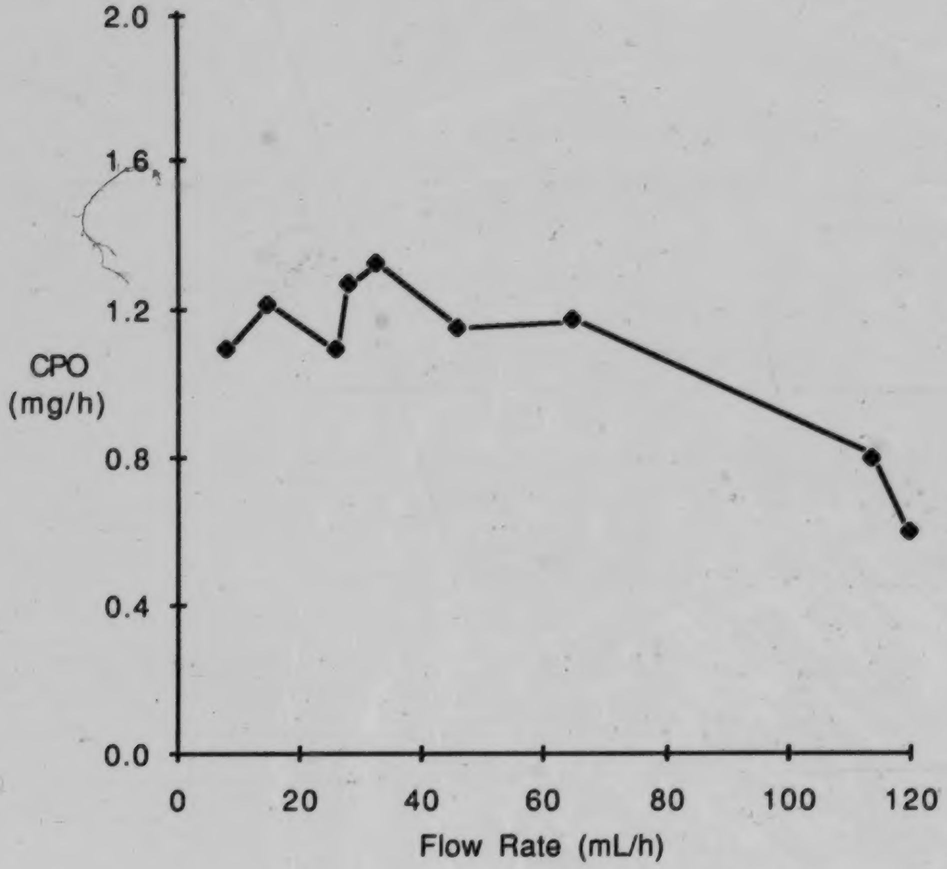
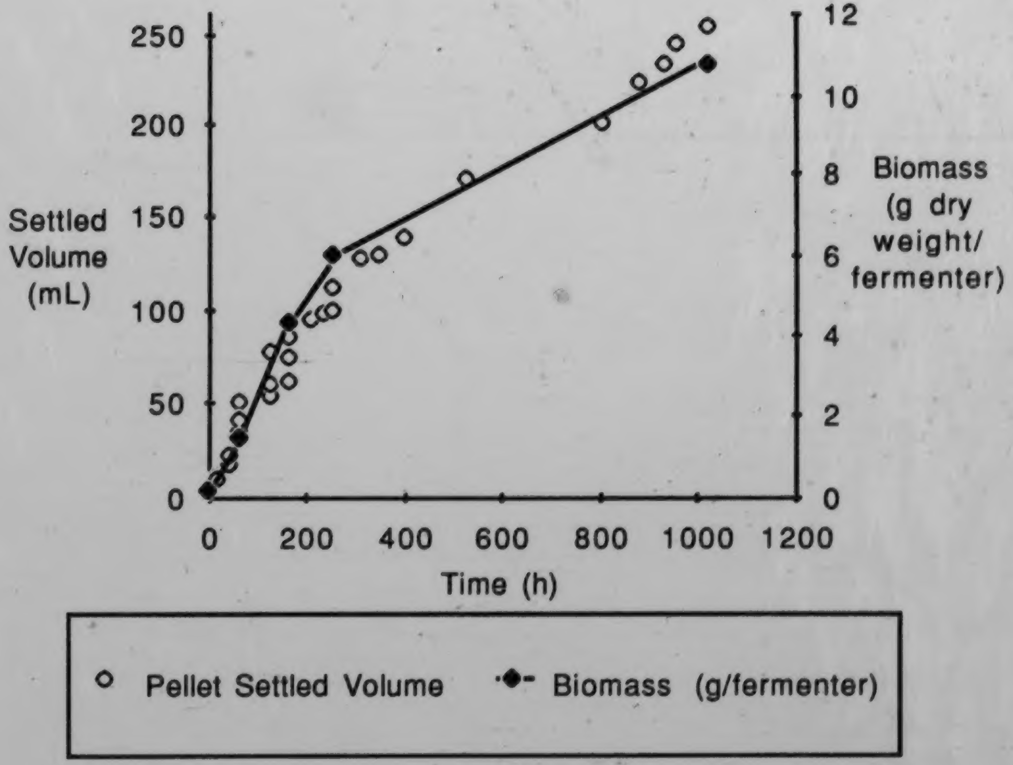

2.0
1.6
1.2
0.8
0.4
0.0
CPO
(mg/h)
0
20
40
60
80
100
120
Flow Rate (mL/h)

Figure 21. Photograph of immobilized *C. fumago* CMI 89362 hyphae after 10 days culture in PFS medium.

After immobilization, 20mL settled volume of immobilized hyphae (0.3 cm diameter) were inoculated into PFS medium containing antifoam A. With 10 days culture the beads had grown to 2 cm diameter and had plugged the downcomer of the fermenter. For the photograph, the fermenter was emptied and a small number of the beads were suspended in distilled water inside the fermenter.

Figure 22. Settled volume of pellets versus biomass in airlift fermenter culture.

Four airlift tower fermenters containing 4% FS medium were inoculated with *C. fumago* CMI 89362 pellets (0.1 cm diameter, 4 mL settled volume, 60 mg dry wt.) and operated as batch cultures. The culture was monitored for biomass increase by following the increase in settled volume of pellets inside the fermenter and harvesting whole fermenters.


Settled Volume (mL)
250
200
150
100
50
0
12
10
8
6
4
2
0
Biomass (g dry weight/ fermenter)
0
200
400
600
800
1000
1200
Time (h)
○ Pellet Settled Volume
-◆- Biomass (g/fermenter)

weight and the settled volume were not linear over an extended period of time.

3.1.5 Large Scale CPO Production

C. fumago CMI 89362 CPO production in the 50 L LH fermenter was carried out with computer monitoring of temperature, pH, and pO_2. In earlier runs at high aeration rates and agitation speeds the fungus grew very well but did not produce high CPO levels. Under moderate aeration (0.6 v/v) and low agitation speed (100 rpm) the fungus grew slowly, in the form of pellets, and high enzyme levels were achieved (approximately 60 mg/L) after 26 days incubation (Fig. 23). Final biomass yield was approximately 300 g of *C. fumago* CMI 89362 pellets.

3.1.6 Other Fungal Fermentations

An attempt to grow the fungus in a 2 L internal loop airlift fermenter (BRL) failed due to fungal pellet adherence to the side and partitioning walls in the fermenter (Fig. 24) caused by the unusual rheology displayed by the fermenter at operational aeration rates (<1 L air/min). Also the same problem eventually occurred when a LH 2 L internal loop fermenter was inoculated with mycelial pellets.

Figure 23. Fermenter profile of large scale CPO production in a 50 L stirred tank fermenter batch culture.

C. fumago CMI 89362 pellets were prepared as described in the Methods section (2.9). The fermenter was set up and medium prepared the same day of inoculation. The fructose was autoclaved separately and added to the salts medium prior to inoculation. The fermenter parameters were as follows: no baffles, 100 % D.O. (=100 rpm & 30 L air/min), 28 °C, pH control to 4.0, using 6N HCl and 6N NaOH, for the first 5 days and to 3.5 for the last 5 days. No pH control was used for the remainder of the fermentation. Samples were obtained aseptically and assayed for CPO levels.

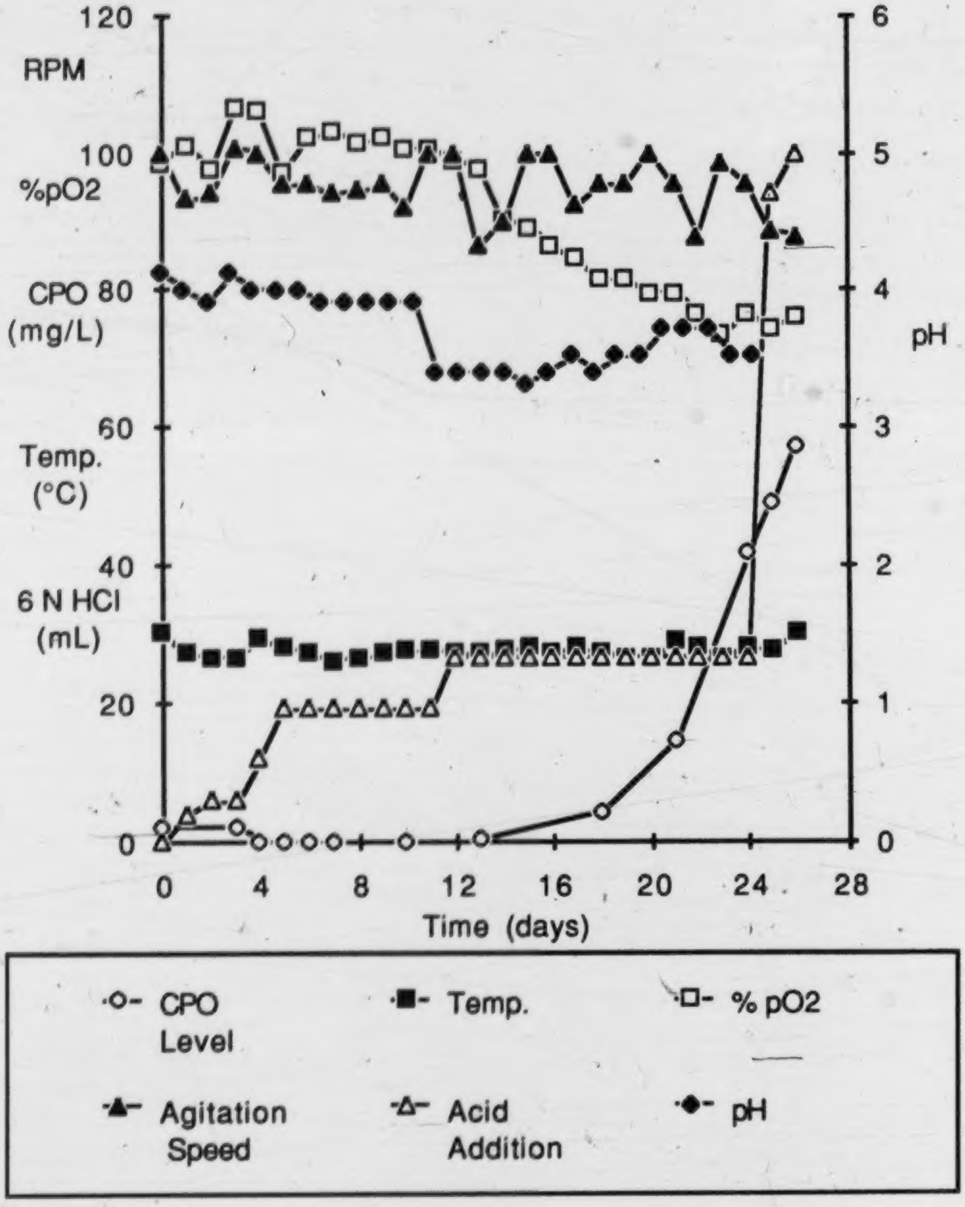

120
RPM
100
%pO2
CPO 80
(mg/L)
60
Temp.
(°C)
40
6 N HCl
(mL)
20
0
6
5
4
pH
3
2
1
0
0
4
8
12
16
20
24
28
Time (days)
-○- CPO Level
-■- Temp.
-□- % pO2
-▲- Agitation Speed
-△- Acid Addition
-◆- pH

Figure 24. Photograph of *C. fumago* CMI 89362 pellets in a BRL 2L airlift fermenter after 21 days culture in FS medium.

After pellet formation in PFS preculture, 10 mL settled volume of fungal pellets (0.1 cm diameter, 60 mg dry weight) were inoculated into PFS medium containing antifoam A. Within 2 days, the fluid dynamics of the fermenter had trapped the pellets along the internal loop partitioning wall where the pellets then continued to grow for the remainder of the experiment.

3.2 CPO POTENTIAL FOR THE TREATMENT OF PHENOLIC WASTEWATERS

Peroxidases have been proposed for the treatment of phenolic containing wastewaters and the spectrum of phenolic oxidizing activity has been determined for HRP (Alberti and Klibanov, 1981). During the HRP study, CPO was also tested for phenol oxidation and was found to be responsible for only low efficiency enzymatic oxidation of phenolic compounds, although no data was presented (Alberti and Klibanov, 1981). However, in Dr. Fedorak's lab, it was found that in the presence of CPO and hydrogen peroxide and in the absence of halides, a phenol solution turned quickly from colorless to a yellow color. This observation indicated that CPO was capable of catalyzing a rapid peroxidase type reaction. Therefore, the following experiments were carried out to determine (1) if CPO was capable of oxidizing a wide variety of phenolic compounds, (2) the optimum conditions for maximum oxidations, and (3) whether or not it has any practical use in the treatment of phenolic wastewaters.

3.2.1 Survey of Phenols for Susceptibility to CPO

Table 3 shows the relative susceptibilities of a variety of phenolic compounds to oxidation by multiple additions of chloroperoxidase and peroxide at pH 3 and 5.5 under conditions similar to those used by Alberti and Klibanov (1981). In the studies by Klibanov and Morris (1981) and Alberti and Klibanov (1981), the peroxidase concentration was varied to maximize the oxidation of the test compound. In this study, the amount of CPO used was kept constant in order to carry out a comparison of the susceptibilities of the phenolic compounds. However this resulted in the choice of an enzyme concentration which was in excess of the amount required to carry out classical enzyme kinetic studies. Therefore few attempts were made to interpret the data in terms of the reaction rate.

The substrates were found to fall into 4 groups based on the amount of oxidation: (1) The

Table 3. Oxidition of phenolic compounds.

	Percent substrate oxidized after 2 h					
	Total		Enzymic*		Precipitate Formed	
Substrate	pH 3.0	pH 5.5	pH 3.0	pH 5.5	pH 3.0	pH 5.5
phenol	81	70	70	62	-	-
o-cresol	88	91	52	60	-	-
m-cresol	>95	>95	72	82	+	+
p-cresol	92	>95	79	64	-	-
2,3-dimethylphenol	64	68	21	50	+	+
2,4-dimethylphenol	72	71	56	54	-	-
2,5-dimethylphenol	73	77	41	53	+	+
2,6-dimethylphenol	62	68	42	52	+	+
3,4-dimethylphenol	>95	>95	80	63	+	+
3,5-dimethylphenol	64	38	57	25	-	-
4-ethylphenol	>95	>95	75	90	+	+
2-chlorophenol	>95	80	82	75	-	-
3-chlorophenol	>95	90	90	85	-	-
4-chlorophenol	>95	>95	88	93	+	+
cyclohexanol	14	3	10	0	-	-
2-methylcyclohexanol	0	0	0	0	-	-
3-methylcyclohexanol	19	7	0	1	-	-
4-methylcyclohexanol	0	0	0	0	-	-

Phenolic reaction mixtures (100 mg/L, pH 2.75, 5.5) were prepared and sampled as described in the Methods section. CPO (5 μg) and H_2O_2 (0.2 μmol) were added at time 0 and at 30 min intervals thereafter. Standard curves for each phenol were prepared and were linear in the concentration range tested. (* enzymic=total - peroxide control.)

chlorophenols were found to be readily oxidized (>95%). (2) Next in susceptibility were phenol and the monoalkyl-phenols. (3) The dimethylphenols were found to be substrates of intermediate susceptibility and (4) the cyclohexanols were found to be poor substrates for this reaction. The low reactivity of the cyclohexanols infers that the aromatic nucleus is a requirement for oxidation. Enzymic oxidation refers to the total phenolic oxidized minus the oxidation due to peroxide. In all cases the enzymic reaction accounted for the majority of the phenol oxidized. Chloroperoxidase was active over a wide pH range (3-5.5) and pH seemed to have no obvious effect on the amount of phenolic oxidized. This was due to presence of excess enzyme in the reaction mixture, which would mask any effect of pH would have on enzyme activity.

Like HRP, chloroperoxidase catalyzed oxidation can result in the formation of precipitates as seen with 7 of the 14 phenolic compounds tested. The formation of precipitates is important to the use of enzymes in wastewater treatment because this will allow the oxidized phenolic compounds to be removed by simple sedimentation or filtration. Precipitates were not formed in all cases but the reaction conditions were standardized to determine the relative susceptibilities of the substrates, not optimized for substrate removal. If reaction conditions were changed so that shock loading concentrations of phenol (>250 mg/L rather than 100 mg/L) were used (see later experiment), the percent phenolic oxidized remained high and a precipitate was formed.

In the survey experiment with various phenolic compounds (Table 3), multiple enzyme and hydrogen peroxide additions were used in an attempt to maximize the oxidation of the phenolic substrate according to the logic used by Klibanov *et al* (1983). As shown in the time course of phenol removal (Fig. 25), each addition (4 in all) resulted in a relatively constant amount of oxidation (20% oxidized) suggesting the enzyme is being inactivated during the reaction. The amount of phenol oxidation catalyzed by peroxide alone was found to vary between experiments but it always remained small (<10%).

Subsequent studies showed that sequential additions of enzyme (Fig. 26a) and the delay

Figure 25. Phenol oxidation using multiple enzyme additions.

Four additions of enzyme (5 μg) and hydrogen peroxide (0.2 μmol) were made at time 0 and every 30 min thereafter to 1 μmol phenol at pH 2.75 or at pH 5.5, as described in the Methods section. The amount of phenol remaining at any time was determined by GC analysis and comparison of peak areas to standard phenol solutions as described in the Methods section.

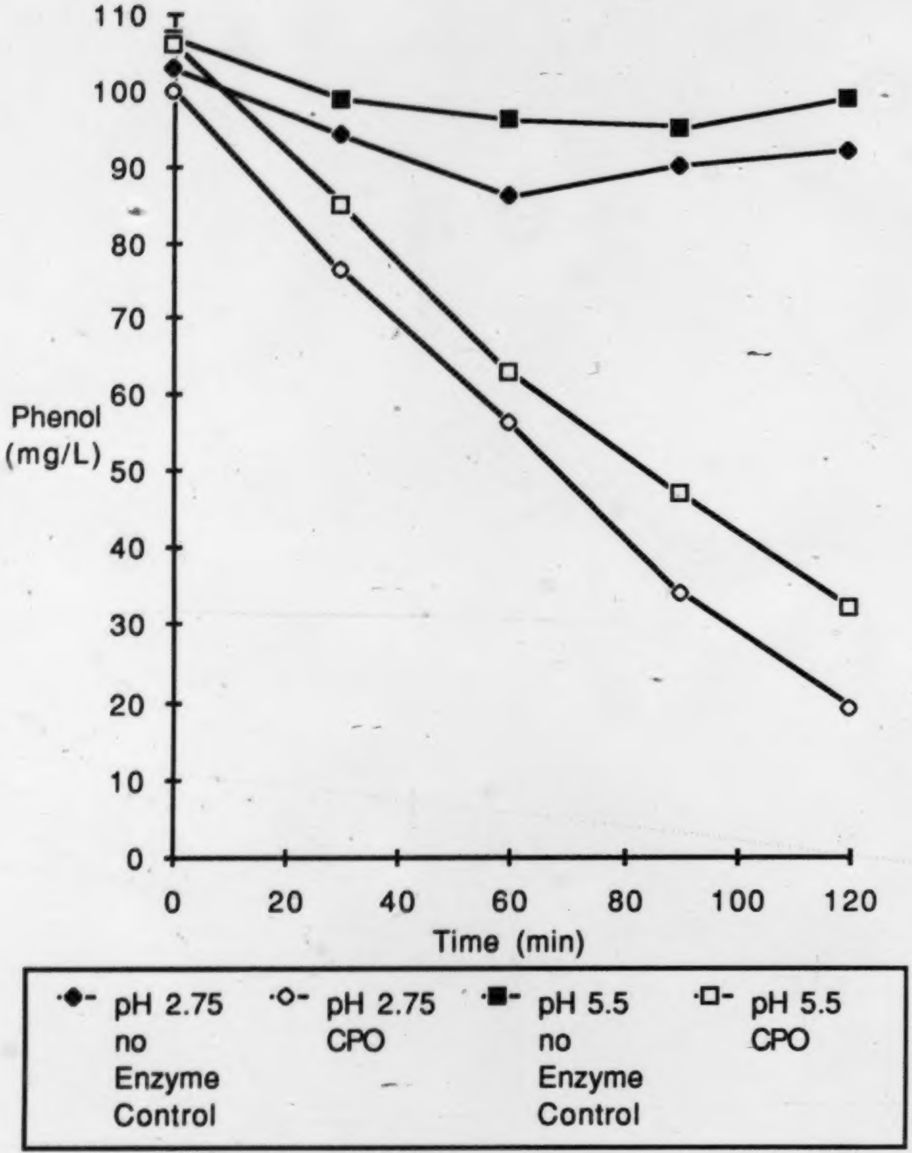
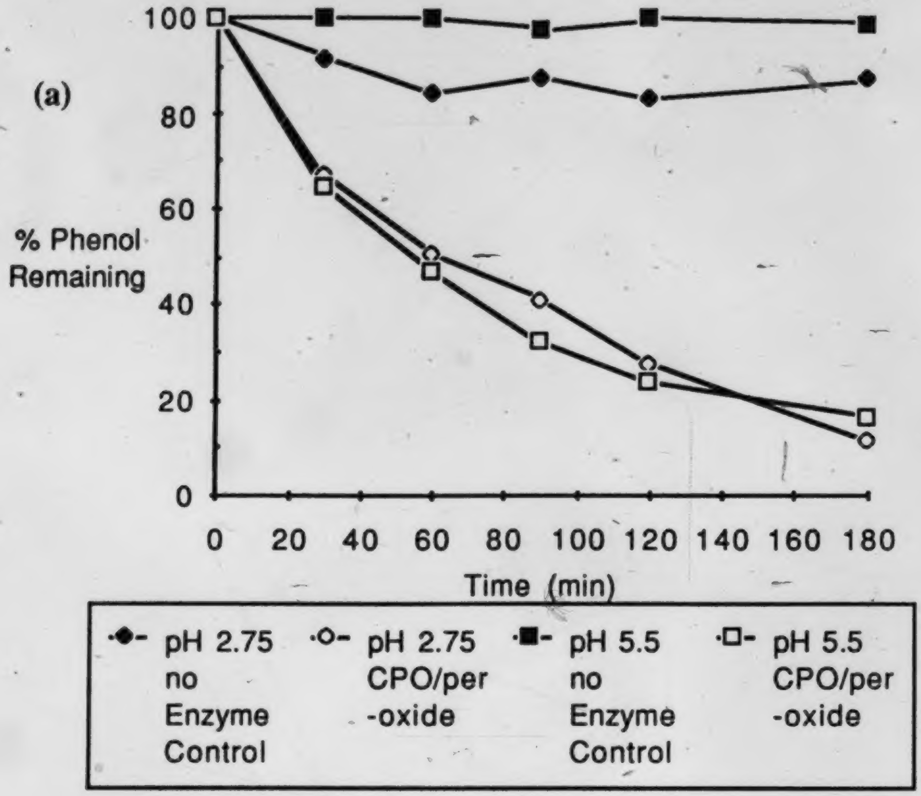

Phenol (mg/L)
0
10
20
30
40
50
60
70
80
90
100
110
Time (min)
0
20
40
60
80
100
120
pH 2.75 no Enzyme Control
pH 2.75 CPO
pH 5.5 no Enzyme Control
pH 5.5 CPO

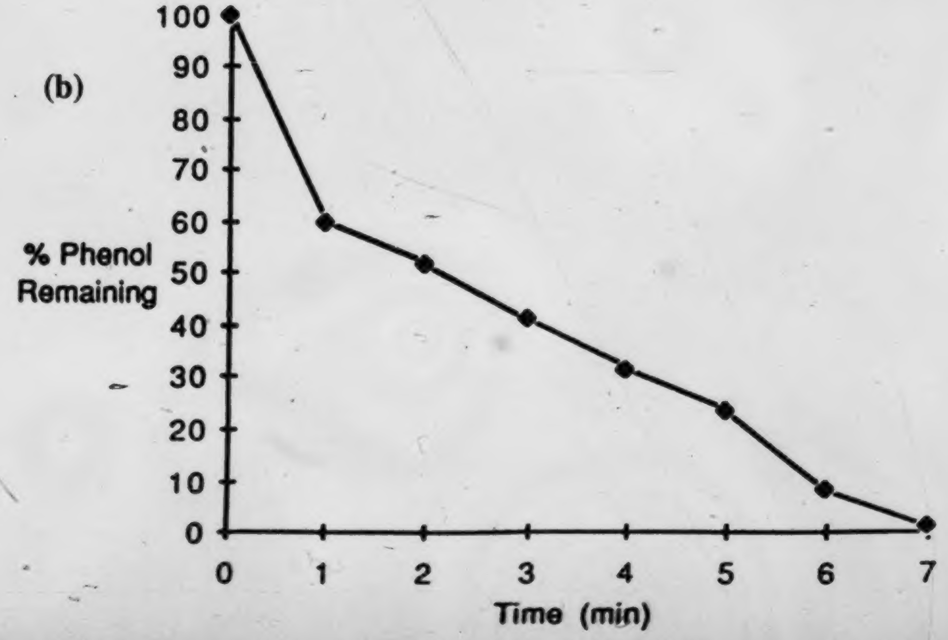
Figure 26. a) Phenol oxidation using a single addition of CPO and slow additions of peroxide.

Reaction mixtures contained a single 5 μg enzyme addition, 5 additions of 0.2 μmol hydrogen peroxide , and 1 μmol phenol at pH 2.75 or at pH 5.5, with 30 min between peroxide additions. Samples were taken and injected into the GC every 30 min to determine the amount of phenol oxidized. Controls (no CPO) were assayed to determine non-enzymatic oxidation at the same times. The time 0 sample, before enzyme and peroxide addition, served as a 0% oxidized sample.

b) Phenol oxidation using a single addition of CPO and rapid additions of peroxide.

A single enzyme addition (5 μg), and 7 hydrogen peroxide additions were added to 1mL of phenol reaction mixture as described above but with 1 min between additions. Samples were taken every min as described in the Methods section and the amount of phenol remaining was determined by comparing peak areas with a time 0 (100% phenol remaining) sample.


(a)
% Phenol Remaining
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
Time (min)
◆ pH 2.75 no Enzyme Control
○ pH 2.75 CPO/per-oxide
■ pH 5.5 no Enzyme Control
□ pH 5.5 CPO/per-oxide


(b)
% Phenol Remaining
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
Time (min)

between additions (Fig. 26b) were not crucial for high phenol oxidation. Both graphs shows the results of chloroperoxidase catalyzed oxidation of phenol at pH 2.75 and 5.5. A single addition of enzyme and 5 additions of hydrogen peroxide, resulted in >85% of the phenol oxidation at pH 2.75 and pH 5.5 over 3 h. With one addition of enzyme and 7 additions of peroxide (Fig. 26b), total oxidation of phenol was achieved after 7 min. This was achieved with a molar addition ratio of H_2O_2:phenol equal to 1.3:1. Also a precipitate was formed that was readily filtered. Despite enzyme inactivation, which will be discussed later, enough CPO was present to catalyze the total removal of the phenol and the straight line response at low phenol concentrations further suggests zero order kinetics. Further studies showed that the catalyzed reaction was completed within 30 s after hydrogen peroxide addition (Fig. 27a) and that 90% of the phenol could be oxidized with a single addition of enzyme and peroxide (Fig. 27b). For this reason the following studies show the percent phenol oxidized as a function of addition number rather than time. From the data shown in Fig. 27b, a turnover number of approximately 2 x 10^4 mol phenol/mol CPO/min was calculated for the phenol oxidation reaction. However, this is likely a conservative estimate since the reaction contained a large excess of enzyme rather than being enzyme limited.

3.2.2 Oxidation at High Phenol Concentrations

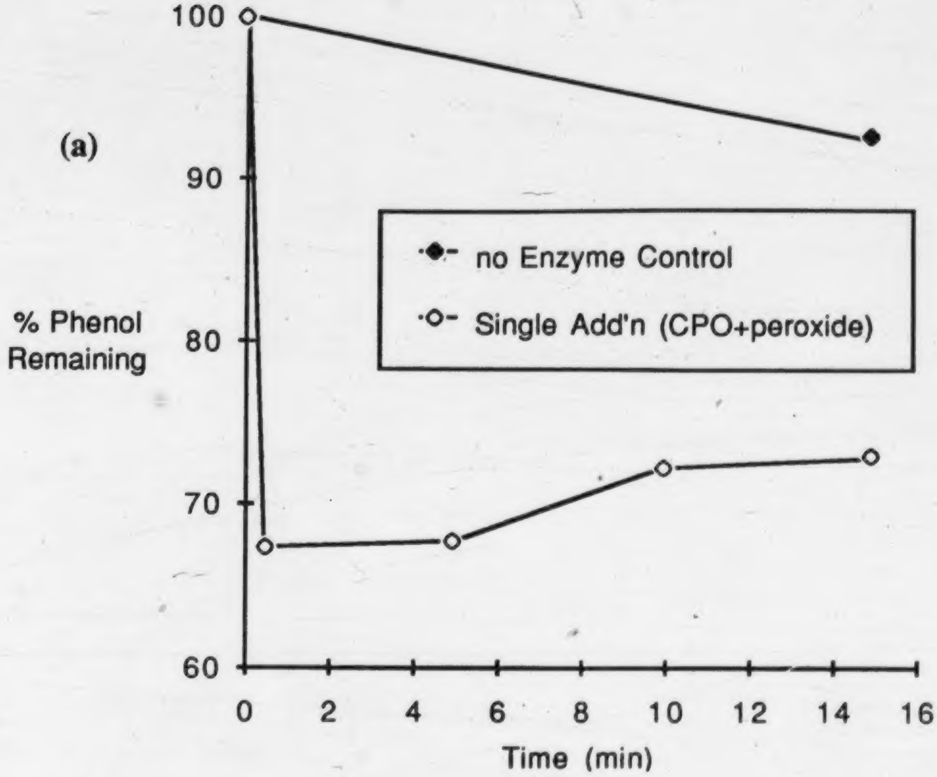
The concentration of phenol in phenolic wastewaters can often be in excess of 4 g/L (Fedorak and Hrudey 1984b). CPO was also able to catalyze phenol oxidations at these shock loading concentrations and these results are shown in Fig. 28. The enzyme was active at high phenol concentrations and was still very effective at oxidizing >70% of the phenol, at phenol concentrations up to 1 g/L. Based on a molecular weight of 42,000 for CPO, the addition of 10 µg of CPO, and assuming 70% oxidation of phenol at 1.0 g/L, the catalytic longevity for CPO was determined to be 3.1 x 10^7moles phenol/moles CPO. Doubling the amount of CPO added did not proportionally increase (approximately 10%) the amount of phenol oxidized (Fig. 29), Figure 27. a) Single enzyme and small peroxide addition.

The initial rate of the oxidation reaction was examined after a single addition of 5 µg of CPO and 0.2 µmol H_2O_2 to 1 µmol phenol. Samples were taken and subjected to GC analysis at 30 s and at t=5, 10 and 15 min and compared to time 0 phenol peak areas to determine the amount of phenol remaining.

b) Single enzyme and large peroxide addition.

To determine if the addition of equimolar amounts of peroxide and phenol to the reaction mixture will result in the same amount of oxidation as multiple additions of peroxide, 5 µg of CPO and 14 µmol H_2O_2 were added to 1 µmol phenol at pH 5.5 in 1mL and samples were taken and analysed by GC as described in the Methods section.

(a)


100
90
80
70
60
% Phenol Remaining
0
2
4
6
8
10
12
14
16
Time (min)
no Enzyme Control
Single Add'n (CPO+peroxide)

(b)

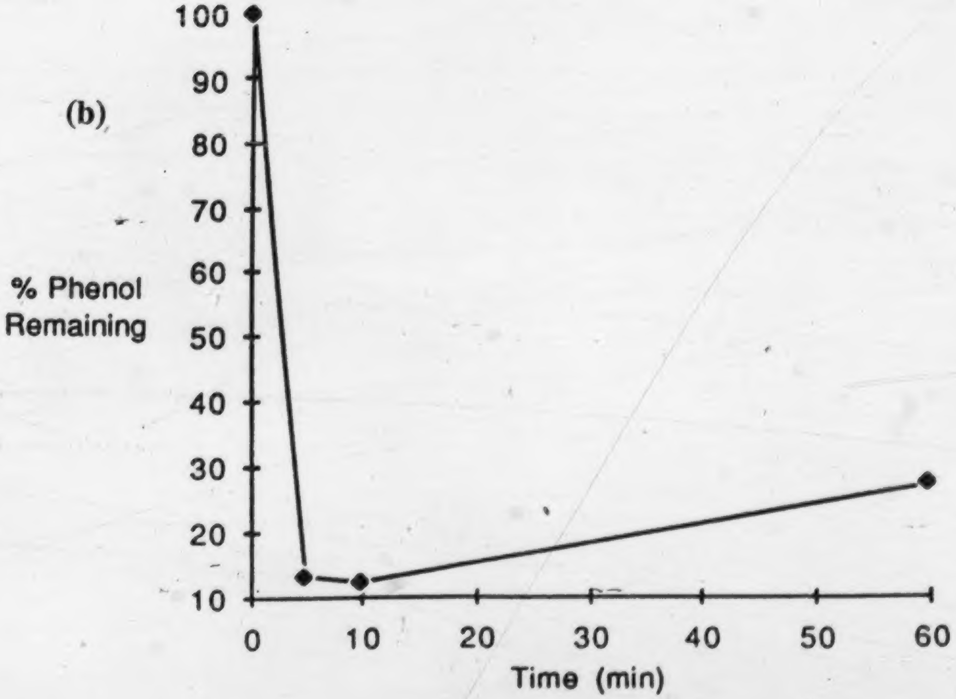

100
90
80
70
60
50
40
30
20
10
% Phenol Remaining
0
10
20
30
40
50
60
Time (min)

Figure 28. CPO catalysis of high phenol concentrations (shock loading).

To determine if higher phenol concentrations affected the oxidation reaction, reaction mixtures (pH 5.5) were prepared with increasing amounts of phenol. A single addition of CPO (5 µg) and multiple additions of proportionally higher amounts of hydrogen peroxide were added to 1 mL of reaction mixture as described in the Methods section. Samples were taken before each addition, diluted with distilled water when necessary and analyzed by GC for the amount of phenol remaining.

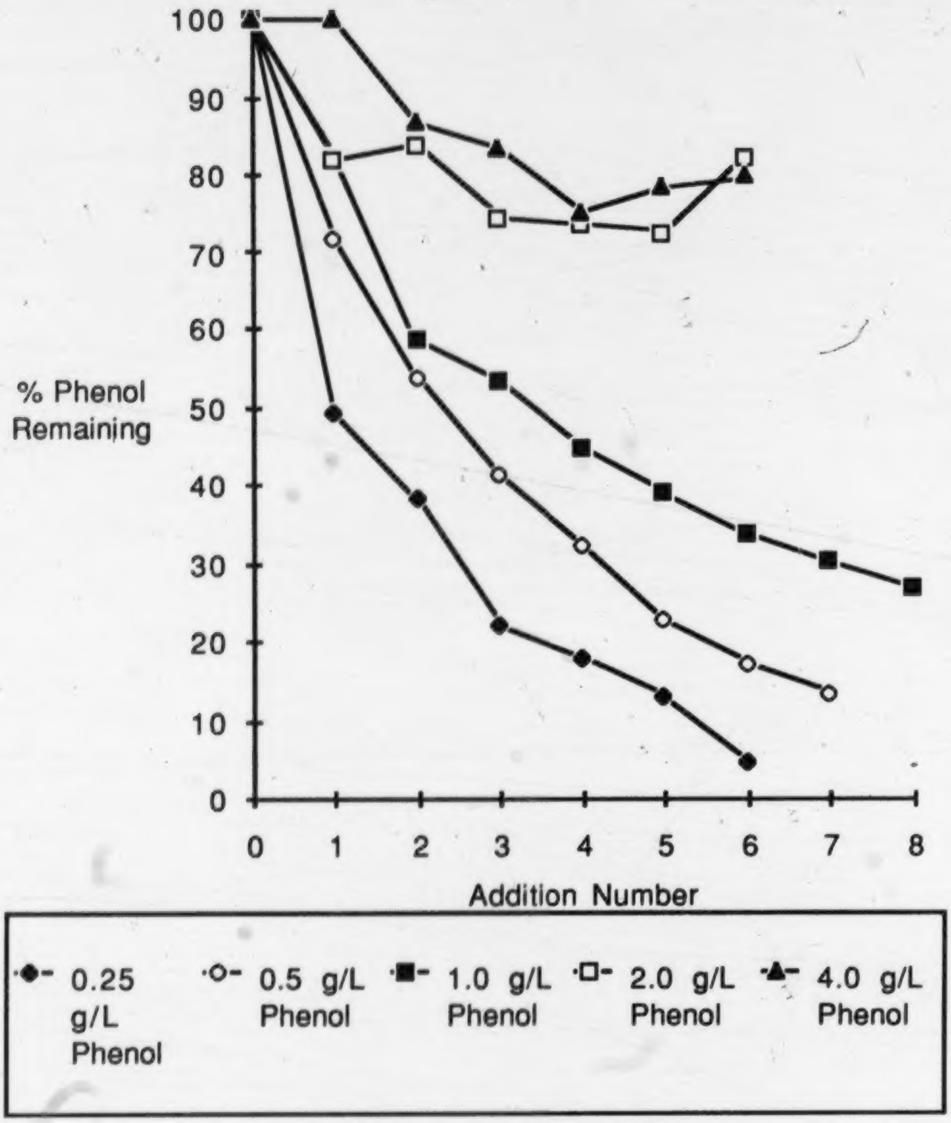
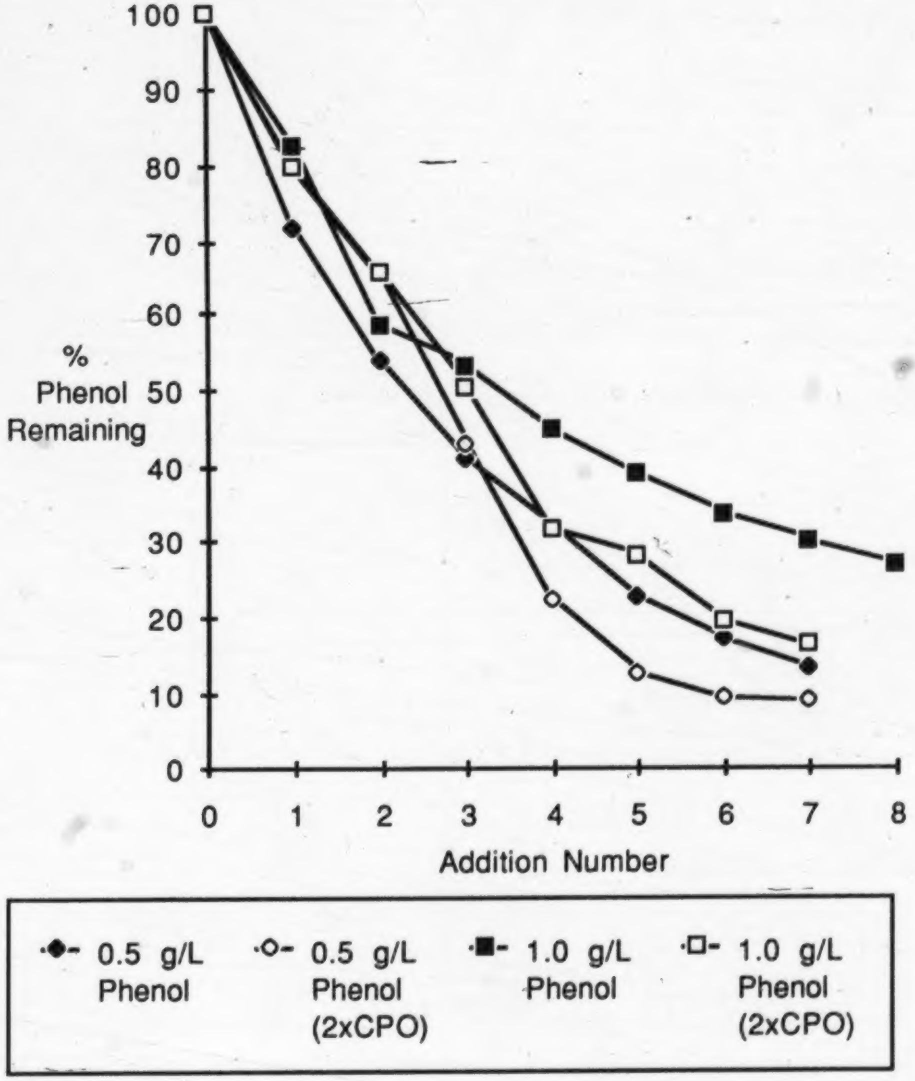

% Phenol Remaining
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Addition Number
0.25 g/L Phenol
0.5 g/L Phenol
1.0 g/L Phenol
2.0 g/L Phenol
4.0 g/L Phenol

Figure 29. Comparison of elevated enzyme levels on phenol oxidation at shock loading concentrations .

To determine if increasing the amount of CPO added at higher phenol concentrations increased the amount of oxidation, reaction mixtures were prepared with 5 µmol and 10 µmol phenol (pH 5.5). A single addition of CPO (10 µg) and multiple additions of proportionally higher amounts of hydrogen peroxide (1 µmol and 2 µmol respectively) were added to 1 mL reaction mixtures. Samples were taken before each addition, and analyzed as described in Fig. 28.


100
90
80
70
60
50
40
30
20
10
0
%
Phenol
Remaining
0
1
2
3
4
5
6
7
8
Addition Number
◆ 0.5 g/L Phenol
○ 0.5 g/L Phenol (2xCPO)
■ 1.0 g/L Phenol
□ 1.0 g/L Phenol (2xCPO)

again indicating that a large excess of enzyme was present.

3.2.3 Further Characterization of Phenolic Transformations

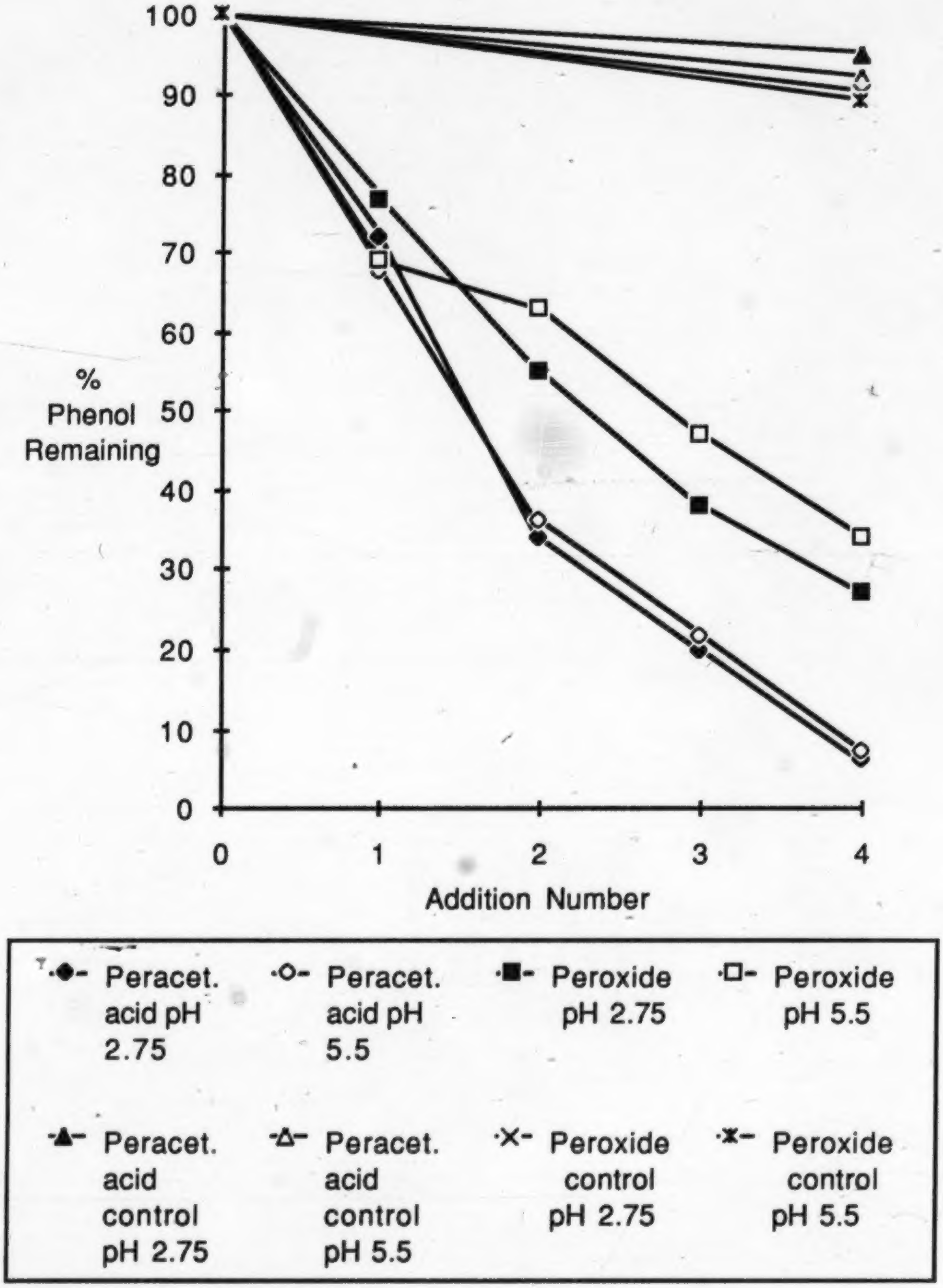
Hydrogen peroxide is not the only co-substrate suitable for CPO catalyzed phenol oxidation. An alternative electron donor, peracetic acid, was found to be a better co-substrate than hydrogen peroxide for the oxidation of phenol (Fig. 30). After 4 additions of peracetic acid <10% of the phenol remained compared to 30% phenol remaining with hydrogen peroxide under similar conditions. Peracetic acid is not a substrate for the catalase activity of CPO which removes hydrogen peroxide from the reaction.

An attempt to determine the effect of pH over the range from pH 2.0 to pH 9.5 on the halide-independent peroxidase reaction was hindered by the fact the reaction mixture contained excess enzyme. Reaction mixtures contained 1 µmol phenol and 5 µg CPO and were prepared with 0.1 M phosphate buffer at various pH values. The reaction was initiated by the addition of 1.0 µmol hydrogen peroxide and sampled at 3, 8 and 12 min. The peak areas obtained from the GC integrator were averaged and compared to peak areas at zero time (average of three values) to determine the amount of phenol remaining. Reaction conditions were adjusted so that after a single addition of CPO and hydrogen peroxide, approximately 80% of the phenol was removed. At lower pH values (2.75-5.0) the amount of phenol remaining was constant (15-20%). Between pH 5.5 and pH 8.5 the amount of phenol remaining after treatment rose steadily to 40%. Above pH 8.5 the enzyme was markedly inactivated. These results are difficult to interpret and the experiment needs to be repeated at lower enzyme concentrations.

The problem of excess CPO was apparent again when a comparison of phenol oxidation was attempted at two temperatures, 21° and 4 °C. A single addition of enzyme (5 µg) and multiple additions of 0.2 µmol hydrogen peroxide were added to reaction mixtures containing 1 µmol phenol (pH 2.75) at the two temperatures. Samples were taken at 30 minute intervals and analyzed as described in the Methods section. Using a single addition of enzyme and multiple

Figure 30. Comparison of peracetic acid and hydrogen peroxide as co-substrates for CPO catalyzed phenol oxidation.

Reaction mixtures contained 1 μmol phenol and 5 μg CPO at pH 2.75 or 5.5. Reactions were initiated by the addition of either 0.2 μmol hydrogen peroxide or 0.2 μmol peracetic acid at 20 min intervals. Samples were taken prior to each addition, and analyzed as described in the Methods section.


100
90
80
70
60
50
40
30
20
10
0
%
Phenol
Remaining
0
1
2
3
4
Addition Number
Peracet. acid pH 2.75
Peracet. acid pH 5.5
Peroxide pH 2.75
Peroxide pH 5.5
Peracet. acid control pH 2.75
Peracet. acid control pH 5.5
Peroxide control pH 2.75
Peroxide control pH 5.5

additions of peroxide at 4 °C, no difference could be detected from a room temperature control. Also, in another experiment, when a single addition of 10 μg enzyme and 2 μmol peroxide was used to treat 2 μmol of phenol at pH 2.75 and 4 °C, the reaction was completed by 30 s with 90% of the phenol oxidized. The results were the same as the room temperature control. Again the presence of excess enzyme has resulted in a masking of any effect temperature may have on the phenol oxidation reaction. For a difference in the kinetic rate, the experiment should be repeated at lower enzyme concentrations. However, this experiment demonstrates, like the Klibanov experiments (Alberti and Klibanov 1981), that under excess enzyme conditions phenol removal is unaffected by pH over a wide range.

The stability of CPO to phenol and hydrogen peroxide together and to phenol and hydrogen peroxide separately were tested and the results are shown in Fig. 31. This was determined by assaying CPO halogenating activity remaining 30 min after exposure to each additions of peroxide, phenol and both substrates together. Neither phenol or peroxide alone affected the amount of halogenating activity but upon addition of both substrate and cosubstrate the amount of activity dropped 50% (pH 5.5) and 80% (pH 2.75) after 120 min.

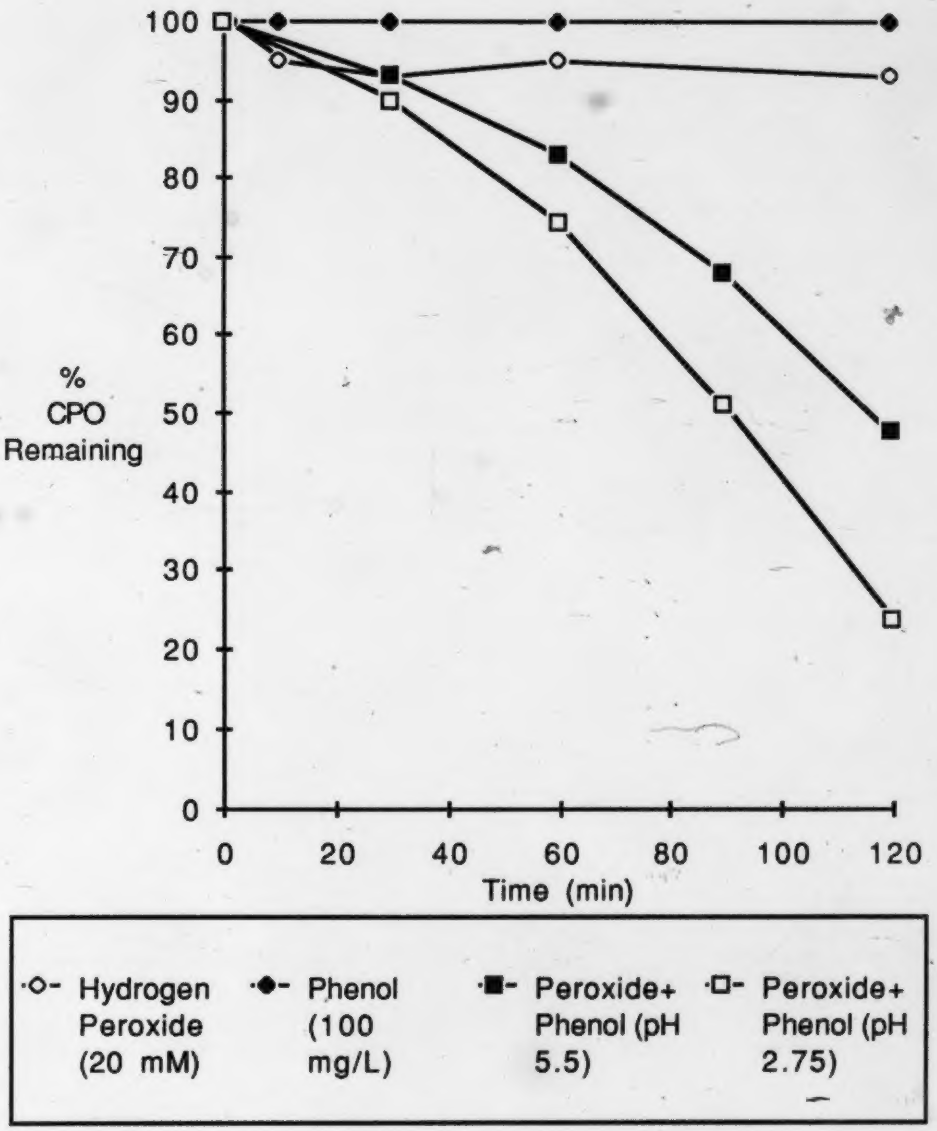
3.2.4 Copolymerization of Phenolic Compounds

One reason put forward for the use of an enzyme-based treatment of phenolic wastewaters was based on the observation that easily oxidized and precipitated phenolic compounds aided in the 'removal' of less reactive aromatic compounds by coprecipitation (Alberti and Klibanov, 1981).

A preliminary study designed to detect copolymerization of phenolic compounds was carried out by treating a solution containing both phenol and benzidine. Neither phenol nor benzidine alone, under the conditions used, 5 μg CPO, 1 μmol H_2O_2, 20 μmol phosphate buffer, pH 2.75, and 1 μmol phenol in 1.0 mL, formed a visible precipitate but when treated together they formed a macroscopic reddish-brown substance that was readily removed from

Figure 31. Stability of CPO in the presence of hydrogen peroxide or phenol, and to the presence of both substrates.

Reaction mixtures contained 1 µmol phenol and 5 µg CPO at pH 2.75 or 5.5. Five peroxide (0.2 µmol) additions were made at 30 min intervals. Separate control reaction mixtures, one containing only 100 µg phenol, pH 5.5 and the other only 20 mM hydrogen peroxide, pH 5.5, but with 5 µg enzyme were run as controls. Samples were taken at t=30, 60, 90, and 120 min and assayed for chlorinating activity as described in the Methods section.


%
CPO
Remaining
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Time (min)
-○- Hydrogen Peroxide (20 mM)
-◆- Phenol (100 mg/L)
-■- Peroxide+ Phenol (pH 5.5)
-□- Peroxide+ Phenol (pH 2.75)

solution by centrifugation. Analysis showed that the amount of unreacted phenol remaining was the same as in the no-benzidine phenol control (15-20%). However the UV absorption at 247 nm, the λmax of benzidine, of the mixture containing both substrates increased after CPO treatment. In the benzidine control there was a 4 fold decrease in the A_{247} nm after CPO treatment. In a similar experiment, 4-chlorophenol (50 mg/L) was used to aid in the oxidation and precipitation of 2,6-dimethylphenol (50 mg/L), a less reactive phenolic. After a single addition of 5 μg CPO and 0.8 μmol H_2O_2, 30% of the 2,6-dimethylphenol remained and no chlorophenol was detected.

3.2.5 Wastewater Treatment Studies

Two industrial wastewaters, H-coal effluent and coal coking wastewater, were tested to determine if CPO could oxidize phenolic compounds in the presence of potential inhibitors such as cyanide (Table 4).

In the first experiment, 1 mL of clarified 2% H-coal effluent (adjusted to pH 5.5) containing 4.2 μg/L cyanide final concentration (Fedorak and Hrudey, 1984) and 76 μmol phenolic was subjected to the addition of 15 μg CPO and 120 μmol H_2O_2. The amount of enzyme catalyzed oxidation observed for phenol, *o*-cresol, *m/p*-cresol were 70%, 28%, 58% respectively. These results indicate a markedly lowered activity if they are compared to the amount of phenolic oxidation (>90%) obtained during the phenolic survey for each of the above phenols even though proportionally increased amounts of CPO and peroxide were added during the treatment. Thus it is possible that some degree of enzyme inhibition has occurred although the exact nature of the inhibitor was not determined.

In the second experiment, free enzyme was used to treat 20 mL of full strength Coal Coking effluent containing 8.3 mg/L total cyanide (Fedorak and Hrudey, 1987). After adjusting the pH to 5.5, the addition of enzyme (500 μg) and 100 μmol hydrogen peroxide resulted in the

Table 4. Composition of Phenolic Wastewaters Treated with CPO

Chemical Constituent	Concentration (mg/L) H-Coal Effluent[1]	 Coal Coking[2]
Organic Carbon	7600	524
Phenolic compounds	7600	410
Nitrite Nitrogen	0.2	0.1
Nitrate Nitrogen	0.8	-
Ammonia Nitrogen	6.4	210
Total Cyanide	**0.21**	**8.3**
pH	7.4	11.8

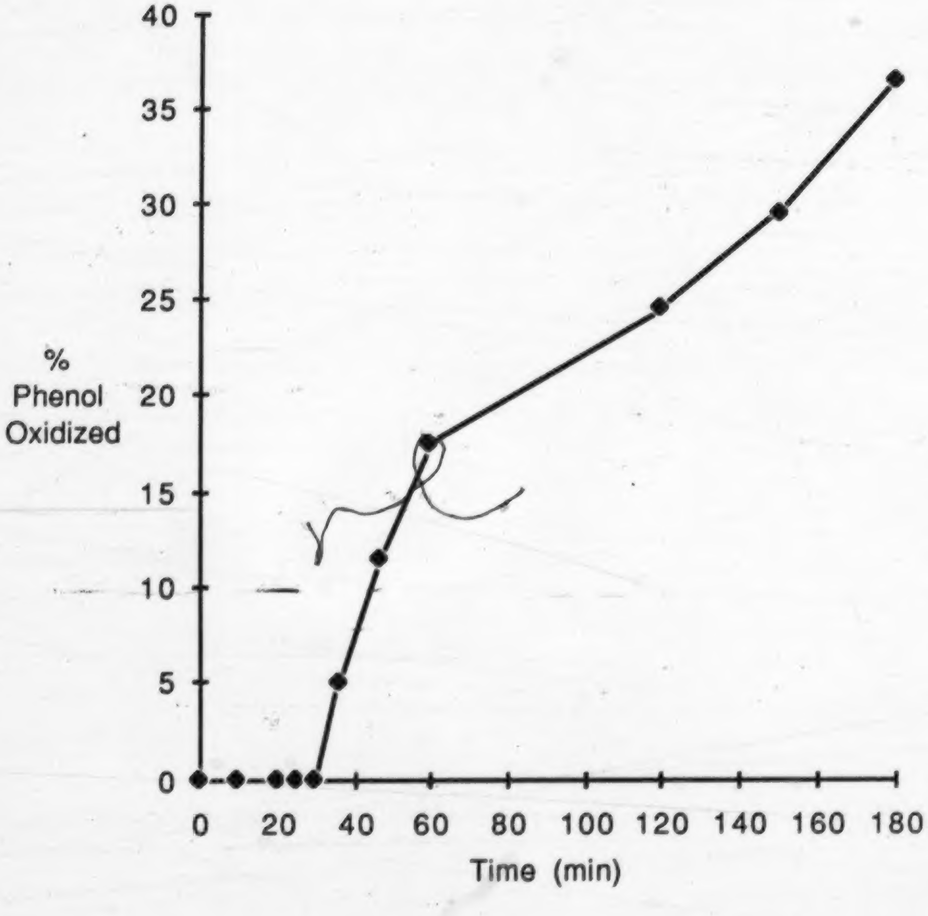
1-from Fedorak and Hrudey, 1984.
2-from Fedorak and Hrudey, 1987.

oxidation of >90% of the phenol and 80% of *p*- and *m*-cresol in 30 s. After treatment the solution was yellow in color and contained a large amount of brown precipitate. In this case the higher cyanide concentration did not seem to inhibit the enzyme catalyzed oxidation of phenol to the same extent as in the H-coal treatment.

Finally, Fig. 32 shows the results of an experiment where a recirculating reaction mixture containing phenol and peroxide was exposed to immobilized enzyme. By using κ-carrageenan entrapped enzyme in this recirculating reactor system, 30% of the phenol had reacted after 180 min of phenol solution circulation. The beads were observed to slowly turned yellow in color especially on the surface where the color eventually went dark brown. The color could not be washed out of the beads. These were the best results obtained to date but they leave much room for improvement. In order to match batch free enzyme treatment results, other methods of enzyme immobilization will likely prove more suitable to a continuous treatment process.

Figure 32. Phenol treatment by immobilized CPO.

CPO was glutaraldehyde crosslinked and entrapped in 5% κ-carrageenan as described in the Methods section. The resulting beads were placed into a pasteur pipette reactor and equilibrated with a phenol containing solution (25 mL, 25 μmol, pH 5.5), which was circulated at 200 mL/h. The hydrogen peroxide (1.0 μmol) was added at 30 min downstream from the reactor and every 10 min thereafter. Samples were taken periodically and assayed by GC for the amount of unreacted phenol still remaining in solution.


%
Phenol
Oxidized
0
5
10
15
20
25
30
35
40
0
20
40
60
80
100
120
140
160
180
Time (min)

4. DISCUSSION

In this project, alternative methods of production to traditional batch culture have been investigated with the hope of developing effective and reliable procedures for the scaleup of CPO production. Also, preliminary studies have been carried out on the potential use of CPO for phenol oxidation and wastewater treatment. The results obtained are discussed under the next two headings which are followed by a cost analysis of the production and treatment costs for CPO production and phenolic wastewater treatment.

4.1 Fungal Growth and Enzyme Production

In the past CPO has been obtained from two strains of *C. fumago*, strain AG 92 (ATCC 16373) by Raistrick (Clutterbuck *et al* 1940) and Hager and coworkers (Hager *et al* 1966a) and strain CMI 89362 by Pickard and coworkers. This study focused on three strains of *C. fumago*, DOAM 137632, ATCC 11925 and CMI 89362. Strain DOAM 137632 was of interest because it was a recent addition to our culture collection and it had shown medium CPO production levels (60 mg/L) in shake flask culture. Strain ATCC 11925 was used due to the high levels of CPO produced in shake flask cultures and unlike the two other strains, it formed firm pellets which readily shed new growth. Also strain ATCC 11925 is believed to be used by Sigma for commercial production of CPO since the enzyme from this source gives a similar isoenzyme pattern to lab prepared samples from this strain (Hashimoto and Pickard 1984). Strain CMI 89362 proved to be the preferred fungus as it produced consistently higher CPO levels, readily formed pellets and formed less pigment and viscosity in culture. Attempts to grow strain ATCC 16373 did not indicate it as the organism of choice.

Some limitations have been placed on developing a standardized inoculum procedure for *C. fumago* culture because the fungus, a hyphomycete, does not readily form asexual spores. Since fungal morphology, growth rate, mycelial yield and substrate utilization rates are dependent on the inoculum size (Ward and Colotelo 1960; Cunningham and Pickard 1985) many aspects of the growth of the fungus may be controlled by using a standardized spore inoculum. The use of spores as source of inoculum would eliminate much of the variability encountered when working with these fungal cultures and to that end Pickard and Hashimoto (unpublished data) have recently developed conditions for the sporulation of strain ATCC 11925. Unfortunately *C. fumago* CMI 89362 still does not readily sporulate and experiments have only begun on modifying the sporulation medium.

Since *C. fumago* CMI 89362 was chosen for use in these production studies, there was considerable importance associated with developing a method of inoculation using homogenized mycelium. It was decided that working with immobilized hyphae or mycelial pellets, generated from homogenized mycelium, would also be desirable because the morphological state of the fungus affects the culture rheology, an important variable controlling mass transfer, which then may affect the final enzyme yield (Anderson 1983). The immobilization of fungal hyphae can be viewed as a form of directed pellet formation where the immobilizing matrix acts as a nucleus for fungal growth.

Once in a bead or pellet form in shake flask culture, the fungus could be maintained in that morphology for extended periods as long as: extreme overcrowding was prevented, low shear forces were used in culture, and the fungus was exposed to nutrient depletion for limited lengths of time. However in airlift fermenter culture with continuous-feed, higher pellet inoculum sizes, 10-30% v/v, were used in 4 consecutive fermenter cultures over a period of two months with little shedding and no sign of reduced CPO synthesis. At the end of each experiment the cultures had nearly taken on the characteristics of a fluidized bed and it was necessary to remove some of the mycelial pellets before starting the next fermentation.

Pellet stability may have been affected by the fermenter culture conditions used, which resulted in low shear forces yet provided better mixing compared to shake flask culture, or by the fact that new growth was constantly stimulated, initially forming a fuzzy layer on the surface of the pellet which later filled in thereby keeping the pellet firm. It should be noted that no attempt was made to optimize the size/diameter of the initial pellet or immobilized hyphae inocula which has been shown to be an important factor in microbial metabolite production (Chien and Sofer 1985). In any event, the original pellet inoculum was ultimately responsible for the production of approximately 800 mg CPO over the four airlift fermenter culture experiments and was discarded only because the diameter of the pellets had increased to a size where physical limitations prevailed.

The growth of mycelial pellets has been shown to follow the cube root law closely for many doublings of the biomass , where $M^{1/3}$=kt + $M_0^{1/3}$ where M= total mass of n pellets, M_0= total mass at time 0, t= time, and k= constant proportional to the growth rate, the size of the growth zone and pellet density (Pirt 1975). The diameter of submerged *C. fumago* pellets was found to obey this observation as they increased linearly with time. In all cases the pellets eventually grew to macroscopic size. In this event the packing of the hyphae may be so compact as to limit substrate permeation except by diffusion (Pirt 1975).

Conditions used for the generation of pellets (low inoculum size) were found to be coincident with the development of less pigment and lower viscosity, and easily separated biomass, culture characteristics suited to downstream purification process. The same could be said for immobilized cells. However the use of the immobilization technique was found to provide a more consistent inoculum because the size range of the beads formed was smaller than the pellets formed by low inoculum. Even though pellets were used primarily throughout this project, the use of mycelial pellets suffers from the following disadvantages: slightly lower enzyme levels achieved by the small inoculum cultures, increased variability, and the extended culture time needed to obtain maximum CPO production. Also, despite all attempts to use the same fungal stocks and inoculum preparation technique, it was observed that a few experiments using pellets towards the end of this project were still plagued by a sudden shift to a low CPO producing morphology characterized by rapid growth, a loose, soft pellet formation, abundant shedding of hyphal fragments, high polysaccharide-high viscosity and foaming. This could be due to the age of the stock inoculum plate and time period used to initial grow the pellets. However pellet formation induced by low fungal inocula has been shown to cause culture variability (Ward and Colotelo 1960). These disadvantages offset the advantage of the simplicity of pellet inoculum preparation to a degree. Experiments still need to be carried out in order to answer these questions concerning pellet inocula preparation and resultant growth.

The CPO levels obtained in semi-continuous culture, by the three immobilized strains of *C. fumago*, were at best only half the values for free mycelium in batch culture and the production period was considerably longer. With repeated transfers, a shake flask culture producing CPO at a constant, albeit suboptimal level, was maintained, thereby eliminating the need to prepare fresh inoculum which may result in batch culture failure for the reasons stated above. Thus by immobilizing *C. fumago*, one can extend the lifetime of the biocatalyst. The use of immobilized cells has already been shown to improve biocatalyst stability (Linko and Linko 1984) as evidenced by prolonged patulin production by immobilized *Penicillium urticae* (Gaucher *et al* 1981).

During the course of the semi-continuous culture experiment, mycelial fragments were being continually sloughed off from the surface of the beads and then removed by the washing procedure used between transfers. The shedding was especially evident with strain ATCC 11925 and to a much lesser extent with strain DOAM 137632 and strain CMI 89362. This observation accounts for the lower increase in bead diameter seen for strain ATCC 11925 and explains why no attempt was made to determine the increase in biomass during the experiment. It also raises the question of the contribution, by immobilized or free sloughed mycelium, to each new round of enzyme production in each passage. No attempt was made to differentiate between the two fungal morphologies present in the culture. However it was believed that the majority of growth was restricted to the beads.

Due to their strictly aerobic nature, the entrapped fungi initially grew towards the surface and eventually covered the surface of the matrix with a dense mycelial layer. A similar pattern of growth has been observed for *Penicillium urticae* growing in κ-carrageenan (Deo *et al* 1983; Deo and Gaucher 1985). Sections of the 3 month old *C. fumago* beads also revealed multiple, dense layers of mycelium exterior to the κ-carrageenan. This was the most likely reason for the eventual decline in CPO levels observed in the semi-continuous culture experiment (Fig. 9) where the increase in bead diameter resulted in reduced oxygen availability, decreased substrate permeation and increased abrasion between beads.

No contamination problems were encountered during the 6 months of semi-continuous culture studies even though the immobilized hyphae were extensively manipulated. This was due to the low pH during growth. The resistance of immobilized cells to contamination in repeated culture has also been reported for bacterial cells (Joshi and Yamazaki 1987).

There were many types of fermenter culture available for the next stage in these production studies but airlift fermenter culture was chosen because of its recent widespread use and many advantages. For instance, immobilized cell stability in stirred tank reactors has been reported as poor (Kloosterman and Lilly 1985) while cellulase production by mycelia of *Aspergillus fumigatus* was lower in agitated culture due to shear damage while air-lift cultures avoided shear effects (Wase *et al* 1985).

In batch airlift fermenter culture using mycelial pellets and immobilized hyphae, the levels of CPO were initially 60-80% and eventually surpassed those obtained in shake flask using free cells (Fig. 6). Batch fermenter profiles were very similar to both the immobilized and free mycelium cultures. It was observed that enzyme production stopped after 15-20 days in fermenter culture. This is apparently not due to carbon or nitrogen limitation and is not restored by a salts feed containing nitrogen, phosphorous, magnesium and iron (data not shown). Experiments to determine what is limiting growth and enzyme production may be answered by carrying out fed-batch fermentations (as described below).

In continuous-feed fermenter culture, low flow rates resulted in effluent levels of CPO equal to the batch run control (Fig. 15) but at higher flow rates enzyme washout occurred. The maximum rate of enzyme production at flow rates below 65 mL/h could be calculated for mycelial pellets in defined fructose-salts medium and was found to be 1.19±0.09 mg CPO/h. Based on this rate of production at slow flow rates, the minimum time required for a conditioned continuously-slow fed culture to match batch system levels would be a 275 h or 11.5 days. After that length of time, any CPO produced can regarded as bonus enzyme. In continuously-fed culture without conditioning the CPO produced was found to initially follow the batch CPO production profile but at higher flow rates production eventually leveled off. Since the unconditioned continuously-fed culture starts with low enzyme levels, the total amount of time to produce the same amount of enzyme would be the same (preconditioning and continuous phase vs continuous phase) but the product would be more diluted and the substrate utilization efficiency would initially be inferior to the preconditioned culture. Thus preconditioning cultures represents a more effective way of producing CPO by reducing the waste of nutrients and producing a more concentrated product.

The large-scale production of CPO was successful in that the yield of CPO was good but the process time was extremely long, 26 days. Most industrial fermentations last from 20-160 h, depending on the enzyme being produced (Lambert 1983). Thus this experiment needs to be repeated using a higher inoculum size which would shorten the time required to reach maximum CPO production.

An alternative method of culture, the fed-batch culture system, was not attempted in this project. Fed-batch cultures have already been used for the stabilization of enzyme levels involved in secondary metabolism resulting in antibiotic production (Vu-Trong and Gray 1986) by allowing additional rounds of RNA and protein synthesis to take place after nutritional downshift had occurred due to medium exhaustion. Fed-batch processes are also used to keep nutrient concentrations low and offer a more efficient use of media than continuously-fed culture (Lambert 1983; Gray and Vu-Trong 1987).

Thus the results obtained during this thesis indicate that: (1) CPO can be produced in a semi-continuous and continuous manner using free pellets or immobilized *C. fumago*, (2) immobilized hyphae and mycelial pellets behaved similarly in airlift fermenter culture; growth was a requirement for enzyme production, (3) the starting time of the feed and the concentration of fructose in the feed could be varied without affecting enzyme production, (4) moderate flow rates (HRT >50h) were best for moderate levels of enzyme production (>30 mg CPO/L), slow flow rates (HRT=65 h) were required to achieve >80% of batch CPO levels (120 mg CPO/L), at flow rates < 65mL/h, a fairly constant rate of CPO production (1.19±0.09 mg CPO/h) was observed, and high flow rates (HRT=9 h) resulted in enzyme washout, (5) traditional batch culture, in fermenters using immobilized cells, was found to still be the method of choice if one considers the simplicity of that system compared to the more involved setup and operational demands of a continuously-fed culture.

4.2 Comparison With Literature Enzyme Production

The semi-continuous enzyme production experiment can be compared to a similar study on protease production by immobilized *Streptomyces fradiae* (Kobuku *et al* 1981). This was carried out in a semi-continuous batch system and conditions were optimized by altering medium composition, gel concentration and sterilizing the gel surface to limit growth. The protease activity of the immobilized mycelia increased over ten reaction cycles, which contrasts with the eventual decline in CPO activity of immobilized *C. fumago* cultures and α-amylase produced by immobilized *B. subtilis* seen over a similar number of cycles (Kobuku *et al* 1981).

Results obtained with immobilized *C. fumago* in continuously-fed culture can be compared to a related study on cellulase production by κ-carrageenan immobilized *Trichoderma reesei* (Frein *et al* 1982), which was carried out in continuously-fed culture over 13 days in an airlift-fluidized bed fermenter column (250 mL) with a flow rate of 2.5 mL/h. Similar to CPO, it was demonstrated that extracellular enzyme could be produced in significant amounts (26

IU/L/h) compared to batch cultures. Also the cellulase system, which used 1/4 strength production medium compared to CPO system's 1/2 strength medium (2% FS), resulted in high specific activities while lowering substrate requirements. Like CPO in continuous-fed culture, cellulase was produced without lag. In the cellulase system, the nitrogen level, in this case the ammonia concentration, was 30% of the level supplied while the reducing sugar content in the effluent fell off precipitously to < 5 µg/mL after 4 days growth. In the case of CPO production, both sugar and nitrogen levels rose and fluctuated during the continuous-fed culture experiments thereby suggesting the need for further fine tuning of the feed composition.

Two other studies related to this project were the production of ligninases by the white-rot fungus *Phanerochaete chrysosporium* using submerged culture (Jager *et al* 1985), semi-continuous and continuous culture (Linko *et al* 1986). Both groups showed that lignin peroxidases could be produced in submerged culture. Linko *et al* (1986) also showed that immobilized mycelium could be used repeatedly for 12 batch cycles. The carbon limited defined medium used for the continuous production of enzyme needed further fine tuning to cycle new growth of the fungus with enzyme production caused by glucose exhaustion. A similar situation exists in this study where continuous chloroperoxidase production by immobilized mycelium requires further feed optimization in order to maximize enzyme production and increase biocatalyst stability.

The immobilized cells used in this study were not resting cells. Growth was required for enzyme production. In shake flasks and batch fermenters, CPO is produced early on in the culture but peaks near the end of the increase in biomass (Pickard 1981) in complex and defined medium. In continuously-fed fermenter cultures CPO eventually reached a fairly constant level in the effluent. This could be explained by: (1) The synthesis of the enzyme requires the active presence of all the complex protein synthesis machinery. Enzyme production would only occur during trophophase when all the necessary precursors are still present in sufficient concentrations. (2) The formation of CPO mRNA would be induced constantly, since fructose is always present in the medium and feed (Axley *et al* 1986). (3) The growth rate was less than maximum (50-80%), which occurred when *C. fumago* was grown on defined medium. Secondary metabolism would occur coincident with the trophophase (Zahner and Kurth 1982) and thus the enzymes responsible for secondary metabolite biosynthesis would be present. Note that extracellular enzyme synthesis can be associated with either the trophophase or idiophase. In continuous culture, a secreted enzyme would be considered growth associated only if it can be maintained at a constant level in the culture supernatant. The fact the CPO levels remained constant in the continuously fed culture gives further support to the requirement for growth.

In contrast, another fungal system (patulin biosynthesis) has already been used as a regulation model for non-growth associated secondary metabolite production. When *Penicillium urticae* was grown on glucose and nitrate or yeast extract, growth cessation was a prerequisite for patulin biosynthetic enzyme production (Gaucher *et al* 1981). Also, semi-continuous production of patulin by immobilized *P. urticae* continued in nitrogen-free medium which suggests the synthesis of enzymes involved in patulin production are less stringent, requiring only a carbon and energy source (Deo and Gaucher 1983). The opposite occurs with *C. fumago*. Growth reduction by nutrient limitation caused a rapid decline in enzyme synthesis (Carmichael *et al* 1986).

Additional speculation on the regulation of CPO production in *C. fumago*, based on information which has been published for enzyme synthesis, will now be presented. Extracellular enzymes may be inducible, partially inducible or constitutive. In addition, fungal exoenzyme genes may have multiple controls, such as the well-defined protease system from *Neurospora crassa* (Priest 1983). The synthesis of many industrially important enzymes are regulated by catabolite repression. In particular the choice of carbohydrate has been found to be important. For example, the synthesis of α-amylase by *Aspergillus oryzae* was repressed by glucose and induced by maltose and the production of cellulase by *Trichoderma viride* was repressed by glucose and induced by sophorose (Lambert 1983). Also *Streptomyces violaceoruber* fructokinase mRNA was found when cultured in the presence of fructose but not with glucose (Martin and Sabater 1981). A similar situation has recently been shown to exist for CPO. Fructose induces the formation of CPO mRNA (Axley *et al* 1986) while glucose represses CPO mRNA formation. Thus a regulatory common denominator for extracellular enzyme synthesis exists although the molecular reasons for catabolite repression appear to vary (Priest 1983).

CPO production may have many controlling elements acting at levels other than transcription (Axley *et al* 1986). For instance, nutrient depletion is not the only growth-limiting factor able to initiate the synthesis of antibiotic biosynthetic enzymes (Gaucher *et al* 1981). There might be some other factor involved which might explain the observation that growth is required for enzyme production. For example, considerable amounts of fructose and nitrate in the culture supernatant when CPO biosynthesis reached a plateau and growth ceased, suggesting that something else is limiting production.

Little is known about the control of secretory pathways in fungi and yet it has been postulated that this may be a rate limiting step in exoenzyme release. For instance, detergents were found to elevate the level of ligninolytic activity (Jager *et al* 1985) suggesting the cell wall may play a part in controlling enzyme secretion (Priest 1983). Although it is evident that secretory pathways vary considerably within the fungi, and if this type of control exists, it may be possible to increase the yield of CPO by using detergents or pleiotropic mutants having aberrant cell walls (Priest 1983). Experiments to determine if this type of control exists in *C. fumago* were not carried out in this project.

CPO production might serve as a model for secretory enzymes involved in the biosynthesis of secondary metabolites and the enzyme expression system may become a powerful instrument for the secretion of foreign gene products (Axley *et al* 1986). Before this can occur, culture techniques for the phasing and dephasing of CPO production need to be developed, since phasing of the culture would aid in the study of the secondary metabolism. The use of glucose, a repressor of CPO mRNA, as a primary carbon source may serve the purpose of restricting CPO biosynthesis to the idiophase. The composition of the growth medium can also be altered to diminish the classical trophophase-idiophase pattern observed in batch fermentation (Vu-Trong and Gray 1982).

The eventual control of industrial fungal fermentations will depend on the development of standardized techniques for the preparation of inocula and measurement of culture variables. Some examples would be substrate levels and their rates of utilization in relation to fungal biomass and product yield. The information gained from such studies may overcome many of the problems that prevent a new enzyme system from being developed to the point where it will successfully compete with existing systems or stand alone in today's marketplace (Witholt *et al* 1985). Production studies must be carried out in order to maximize product yield, product concentration and the rate of product formation while overcoming special problems present in fungal fermentations such as filamentous morphology and eukaryotic growth kinetics. There should also be consideration for the cost effectiveness of the process, and the scale-up of enzyme production will involve the development of the expertise in the handling of the cells responsible for the synthesis of the enzyme.

4.3 Phenolic Compound Transformations

Enzymic methods have been proposed for the removal of aromatic compounds from high strength industrial wastewater. Many enzymes, including CPO, horseradish peroxidase, polyphenol oxidase, cytochrome oxidase, immobilized haemoglobin and lactoperoxidase have been reported to show promise in the oxidation of phenolic compounds. In all of these studies it was important to show that the enzyme catalyzed oxidation was (1) effective against a wide variety of phenols, (2) the products of the oxidation formed precipitates, (3) the oxidation took place over a wide range of reaction conditions, (4) actual wastewaters were successfully treated and (5) that the products formed were determined and analyzed for toxicity (Maloney *et al* 1986). This analysis protocol has been recently completed for HRP, with the results from the first 4 steps giving promise, while the 5th step placed limitations on the use of an enzymic system (Maloney *et al* 1986). In this project the first 4 steps in the analysis protocol were addressed.

In this study, the conditions used to determine the relative susceptibilities of phenolic compounds involved multiple additions of peroxide and enzyme, which was the method used by Klibanov et al (1983). The reaction conditions used by Klibanov and coworkers (Klibanov *et al* 1983) were selected on the following basis. (1) At a phenol concentration of 1 µmol/mL, 1 unit of peroxidase/mL and 2 mM peroxide were required for 99% phenol oxidation. (2) Peroxide is required in stoicheometric amounts. (3) The amount of enzyme that would be inactivated by the reaction products would be proportional to the initial phenol concentration. Some of the following observations also contributed to the development of reaction conditions. They found the removal efficiency was higher when they used multiple additions of enzyme. They observed that a decrease in enzyme concentration resulted in a decrease in substrate (*o*-dianisidine) oxidation however this could be offset by an increase in treatment time (Klibanov and Morris 1981). As a result, Alberti and Klibanov (1981) chose an enzyme concentration that would require a treatment time of 3 h although it was not made clear when the multiple additions of enzyme took place during that time. In this study, an attempt was made to develop a standardized method of estimating the amount of phenolic compound oxidation by using a rapid GC analysis of samples taken immediately before each addition of enzyme and co-substrate. Like Alberti and Klibanov (1981), multiple additions of enzyme and peroxide were used in order to maximize the amount of oxidation. It was hoped that these reaction conditions would establish the relative susceptibilities of the phenolic compounds to CPO oxidation.

Thus the initial survey study, using excess enzyme, found that the halide-independent activity of chloroperoxidase catalyzed the oxidation of a variety of substituted phenolic compounds. This activity was comparable and in some respects superior (Table 5) to the proposed HRP/H_2O_2 system. The results for HRP and PPO are under optimum conditions where the amount of enzyme and peroxide have been adjusted for maximal oxidation of each phenol.

The CPO oxidation efficiencies were determined from experiments where a standardized addition of enzyme and cosubstrate were used to show that CPO was capable of high oxidation of a variety of phenols. CPO was more effective than HRP in removing 3-chlorophenol and 2-methylphenol but inferior in the removal of 2,3 and 2,6-dimethylphenol. CPO was not as effective in oxidizing some phenols as PPO or HRP, but then the reaction conditions were not optimized for high efficiency. Conditions were standardized to determine the relative susceptibilities of the substrates to enzymic oxidation. LPO was largely ineffective against most phenolic compounds except benzidine. Another enzyme, fungal laccase, has the ability to oxidize dimethoxyphenols and 2,6-dimethylphenol (>90% removed) but phenol (3% removed), the cresols (ortho-26%, meta-10%, para-47% removed) and 2-chlorophenol (8% removed) were poor substrates (Shuttleworth and Bollag 1986).

In many respects two of the enzymic systems, HRP and CPO, were found to be quite comparable. Like HRP, CPO was unaffected by lowered temperature and it was highly active over a wide pH range (pH 2 -7). However this was due to the fact that excess enzyme was used in the reaction mixture, which then masked any effect temperature and pH would have on the enzyme catalyzed reaction. The broader temperature range of the enzymic systems has been regarded as a advantage since existing outdoor microbial treatment processes have temperature restrictions (Alberti and Klibanov 1981). The pH range of CPO's peroxidase activity could compliment HRP if the two enzymes were used in combination, thereby extending the enzymic system's oxidizing activity into the acid side of the pH spectrum.

As with HRP (Klibanov *et al* 1983), CPO was active at high phenol concentrations (Fig. 27). A single addition of enzyme (5 μg) was used for all the experiments at elevated phenol

Table 5. Comparison of Removal Efficiencies of Selected phenolic compounds.

	% Phenol Oxidized (Removal Efficiency)				
Phenolic	CPO	HRP[1]	LPO[1]	PPO[2]	FL[3]
2-methoxyphenol	88	98.0	32	65	92
phenol	81	87.6	0.0	100	3
2-methylphenol	88	86.2	-	95	26
3-methylphenol	>95	95.3	15	100	10
4-methylphenol	92	98.5	-	-	47
2-chlorophenol	>95	99.8	13	94	8
3-chlorophenol	>95	66.9	-	96	7
benzidine	66	99.9	91	-	-
2,3-dimethylphenol	64	99.7	-	70	-
2,6-dimethylphenol	62	82.3	-	-	95

1- from Alberti and Klibanov 1981.

2- from Atlow *et al* 1984.

3-from Shuttleworth and Bollag 1986.

- = not done

CPO=chloroperoxidase

HRP=horseradish peroxidase

LPO=lactoperoxidase

PPO=polyphenol oxidase

FL=fungal laccase

levels but the amount of co-substrate added, hydrogen peroxide, was increased proportionally to the substrate concentration. Doubling the amount of CPO added to the reaction mixture did not proportionally increase the amount of phenol oxidized (Fig. 29), suggesting that there was an increase in enzyme inactivation by the reaction products, or the possibility of the enzyme coprecipitating with the large amount of polymerized product formed at higher phenol concentrations.

Further experiments confirmed CPO shared the same lack of stability to the reaction products as HRP. Klibanov *et al* (1983) reported that low peroxidase concentrations resulted in the incomplete oxidation of phenol. Further enzyme additions were required to overcome enzyme inactivation. As in this study (see Fig. 31), they followed the loss of activity by direct assay of samples before and after the reaction. CPO was stable to 20 mM peroxide and to 1 μmol/mL phenol, pH 5.5, separately but not to a mixture of the two substrates in the reaction mixture. Klibanov *et al* (1983) postulated that the HRP inactivation was caused by phenoxy radicals interfering with the active site, and a similar situation probably exists for CPO. The difference in enzyme inactivation due to the pH was difficult to interpret since enzyme was in excess.

CPO was able to polymerize and copolymerize phenolic compounds. This duplicated the findings of Alberti and Klibanov (1981), which showed that the products of the reaction eventually polymerized and could be removed from solution by centrifugation or filtration. Like HRP, CPO also was shown to be able to copolymerize compounds, benizidine and phenol. The copolymerization of the phenolic compounds has been considered an advantage to the enzyme system (Alberti and Klibanov 1981; Atlow *et al* 1984).

However, unlike HRP, CPO has a catalytic longevity of 1.3×10^7mol phenol/mol CPO, which is three orders of magnitude greater than the one published for HRP $=1 \times 10^4$ mol phenol/mol HRP(Klibanov *et al* 1983). Another difference to note between the HRP and CPO systems is the length of time for the treatment. The way the reaction conditions were arranged, HRP treatment required 3 h or more while it has been shown that with excess enzyme and rapid GC analysis of the reaction mixture, total oxidation of phenol by CPO could occur in less than 10 min.

There are some disadvantages to the various enzymic treatments. Polyphenol oxidase treatment would have the added cost of continuous sparging and rapid mixing in order to bring the relatively expensive cosubstrate (oxygen) in contact with the enzyme (Atlow *et al* 1984). A disadvantage to the CPO system would be the cost, if commercially produced enzyme was used (see cost effectiveness of CPO). The opposite would be true for haemoglobin. In its case the low cost would be an advantage (Chapsal *et al* 1986).

Another concern would be the nature of the products formed during the enzyme catalyzed reaction. As mentioned earlier, there was no attempt made in this project to address the concerns raised by the 5 step in the analysis protocol (reaction product analysis). Like HRP (Maloney *et al* 1986), there are different reaction products resulting from CPO treatment. Depending on the substrate and its concentration, precipitates or soluble colored products are formed. Also, since CPO has a potent chlorinating ability (Geigert and Neidleman 1986), with a maximum near pH 3 and a broad peroxidase activity (pH 3-5), activity competition may occur, especially in wastewaters containing halide ions at low pH. This explains why experiments in this project were carried out at both pH 3 and 5. The results obtained herein indicate that there could be reason for concern on the applicability of CPO to wastewater treatment. More experimentation could determine how much peroxidase activity would occur in the presence of halide. It will be important to differentiate between the peroxidase and chlorinating activity of the enzyme, especially at pH 5.5, because the halogenating activity of the enzyme may limit phenolic oxidation and result in the formation of highly toxic halogenated aromatics (Maloney *et al* 1986). Another problem may occur at low substrate concentrations. Maloney *et al* (1986) found that, dependent on the substrate substituent groups, chlorinated phenols are transformed into dioxins and chlorinated furans. Also 50% or more of the products remained in solution.

HRP (Klibanov *et al* 1983) and PPO (Atlow *et al* 1984) have been used successfully to treat actual wastewaters. In this study, CPO was able to oxidize greater than 80% of the phenolic compounds in a coal coking wastewater, despite the presence of a potent inhibitor of peroxidases, cyanide. HRP also shows low inhibition by cyanide at high concentrations of phenol, possibly due to competitition for the active site of the enzyme (Klibanov *et al* 1983). With the coal coking wastewater, a more complex situation may exist due to the difficultly in measuring free cyanide, and the presence of nitrate. Total cyanide was determined for the wastewater (Fedorak and Hrudey 1984b). Since cyanide readily forms soluble metal complexes (Knowles and Bunch 1986), the amount of free cyanide that would be able to interact with CPO maybe much lower than indicated. Also nitrate has been shown to be a competitive inhibitor of cyanide binding to CPO (Lambeir *et al* 1983). Therefore no conclusion can be made on the apparent discrepancy in the effect of cyanide on CPO during wastewater treatment.

Immobilized enzyme systems have been developed for the treatment of phenols (Shuttleworth and Bollag 1986) and as multifunctional halogenating agents (Itoh *et al* 1987; Laane *et al* 1987). Immobilized CPO was used in this project to treat a recirculating phenol solution. However the method chosen for immobilization, gel entrapment, was found to be inadequate for the purpose. In contrast, bromoperoxidase was successfully entrapped in a number of matrixes, due to its high molecular weight (Mwt.= 790,000). This recent research also showed the poorest activity occurred when bromoperoxidase was immobilized in κ-carrageenan (Itoh *et al* 1987). Further research should lead to improvement on the results (30% phenol oxidized in 3 h) obtained with CPO in κ-carrageenan and perhaps another type of matrix or method of immobilization will lead to the development of an effective wastewater treatment system.

4.4 Cost Effectiveness of CPO

The production of plant enzymes is generally less cost-effective than microbial enzymes

(Chibata and Tosa 1981; Klibanov *et al* 1983) although alternative methods like cell culture and recombinant DNA techniques are now being developed to produce large quantities of enzyme (Neidleman and Geigert 1986).

A rough estimation of the cost of crude HRP as part of industrial process has been calculated (Alberti and Klibanov 1981) and has been shown to be competitive with existing systems of treatment (Neufeld 1984) such as FMC Fenton's reagent or a coal conversion treatment train (Table 6). For the treatment of 1000 L of wastewater containing 2 mM phenols, 5-10 g CPO would be required. Using crude enzyme, produced in the lab using 4% FS medium, the treatment of 1000L of phenolic wastewater would cost approximately $40.00. This cost estimate for CPO must be qualified since the optimal concentration of enzyme was not determined for the oxidation reaction. Excess enzyme was used in the reaction mixture and therefore the cost estimate may be high for CPO.

The cost of using commercial enzyme at todays market cost appears to be ridiculously high but then the cost today has been determined by supply and demand. Should enzymes find a use in wastewater treatment, CPO, provided by *C. fumago* grown in fructose salts and rented fermenters, could be a viable economic alternative to HRP, using the techniques of culture and fermentation developed in this project, for the oxidation of phenolic compounds.

4.5 Concluding Remarks

In summary, the major concern of this project was to show the feasibility of CPO production in a semi-continuous and continuous fashion. This involved bioreactor redesign and the development of immobilization methods for *Caldariomyces* sp. and CPO. In addition, this project attempts to bring about some understanding of the growth and morphologies exhibited by *Caldariomyces fumago* in shake flask and fermenter culture during CPO production. The use of immobilized *C. fumago* for CPO production was found to have many advantages including ease in inoculum standardization, biocatalyst stability, increased resistance to

Table 6. Cost Comparison of Various Enzymes Proposed for Phenol Oxidation.

Enzyme	Source	Purity (Rz)	Enz. Cost ($/g Sigma)	Enzyme (mg/L)[1]	Enz. Cost ($/1000 L)[2]
HRP	Plant	0.6	46.72	1.25	58.40
CPO	Fungi	0.8	39,090.00	10.0	390,900.00
CPO[3]	Lab	1.4	17,195.00	10.0	171,950.00
CPO[4]	Lab	crude	20.75	10.0	207.50
CPO[5]	Lab	crude	4.00	10.0	40.00
PPO	Fungi	----	523.00	100	52,300.00
LPO	Mammal	0.8	1,843.00	11,000	20 million
Hb	Human	cryst.	7.85	6,800	53,380.00

Industrial Process [6]	Material/Treatment	Cost ($/1000L)
FMC	Fe^{+2},H_2O_2	0.84
Coal Conversion	Biooxidation	57.00
	Phenol Extraction	19.00
	Whole Treatment Train	26.00-114.00
	Activated Carbon	23.00-45.00
	Ozonation	38.00

Enzyme costs were taken from the Sigma 1987 product catalogue. The amount of enzyme required to treat one liter of wastewater was calculated from published data on horseradish peroxidase, lactoperoxidase (Alberti and Klibanov 1981), polyphenol oxidase (Klibanov 1983), and hemoglobin (Chapsal *et al* 1986). The cost of treatment for 1000L of wastewater assumed 100 % efficiency in scale-up.

FMC= Industrial Chemical Group, FMC Corporation, Princeton, New Jersey.
1 -Amount of enzyme required to treat 1 L 2 mM phenols.
2 -1000L wastewater containing 2 mM phenols.
3 - Lab Cost includes fermenter hardware, medium and salaries.
4 -Lab Cost for shake flask experiment. Includes cost for media, equipment and labour.
5 -only medium cost.
6- from Neufeld (1984).

contamination, stabilization of culture rheology. CPO production is not hampered by problems of low productivity encountered by most immobilized whole fungal cells (Deshpande *et al* 1987) and the immobilized cells require no special treatment other than the removal of free cells between fermentations. Techniques for the cost-effective production of gram quantities have been developed for laboratory scale fermenters. In addition, the extracellular nature of CPO and lower pigment and viscosity levels obtained in the immobilized hyphal culture would be in keeping with industrial process demands for simplified recovery and purification, and further lowering the cost of the enzyme.

The potential for CPO catalyzed phenolic wastewater treatment was found to compare favourably with other proposed enzymic systems, when excess enzyme was used. However the same problem, potential formation of extremely toxic products, exists as for all enzyme mediated removal systems and until the safety of this system is assured it will prevent their implementation. CPO will only be a cost-effective alternative to HRP if methods are scaled up to pilot plant sized fermenters and would be aided by the screening for pigment-less mutants.

It was not the intention of this project to determine the physiology of CPO production but it was hoped that some information could be gained during the course of the production experiments towards a better understanding of the conditions required for the accumulation of consistent high levels of CPO in the culture supernatant. This project on CPO production by immobilized fungal hyphae may do little to change the present state of fungal fermentation but perhaps it represents a small step towards that goal. However this thesis fails to conclusively show that CPO catalyzed phenol oxidation would be a safe method for the treatment of wastewaters while perpetuating the concept of the potential need of large quantities of CPO. More work is required especially in the area of enzyme immobilization and actual wastewater treatment.

4.6 Further Studies

a) Phenol Oxidation with Immobilized CPO

Of primary concern should be the determination of the optimum enzyme concentration required for complete phenolic compound oxidation. This would involve altering reaction conditions so that substrate would be in excess to the enzyme.

Other initial experiments could compare different immobilization techniques: e.g. crosslinking (glutaraldehyde), entrapment (carrageenan), covalent bonding (controlled pore glass), adsorption (celite), and co-immobilization with glucose oxidase for *in situ* cosubstrate generation. This would involve the development of a suitable assay system for the immobilized enzyme, detecting either the chlorinating or peroxidase activity of CPO. This may require the use of a spectrophotometric flow cell. The basis for comparison should include half life of the enzyme and the specific activity immobilized per unit immobilization substrate. An important feature of enzyme immobilization is to develop a process which increases the half life of the enzymes. The enzyme's longevity is more important than the initial activity (Bucke 1983). The use of crude versus pure enzyme could also be investigated at this time. If crude enzyme, minus pigment, could be used it would increase the cost effectiveness of the CPO system. Also various protein stabilization techniques could be tried including BSA addition, and glutaraldehyde crosslinking.

Once a method of immobilization has been chosen experiments can be done to characterize the immobilized enzyme system. Immobilized enzyme stability, temperature, pH, longevity, number of reuses, potential for recharging, effectiveness of phenol oxidation using synthetic and actual wastewaters, stability to organic solvents, effect of trace heavy metals and cyanide on CPO activity, kinetics, etc., are just a few of the variables that could be determined.

Additional experiments that could be done include finding an effective 'reaction stopper', and determining the biodegradability of the reaction products using Dr. Fedorak's anaerobic cultures. The observation that copolymerization occurs with benzidine and phenol could be examined further with other combinations of phenols. The nature and toxicity of the reaction products in each case should be determined.

Finally, continuous treatment of wastewaters using immobilized CPO should be attempted. This would involve the design of a small scale benchtop bioreactor (approximately 1L) for use in such an application and the study of an efficient means of co-substrate addition.

b) CPO Production Studies

Many items of interest still remain unexplored in the area of fungal growth and its relationship to CPO production. First and foremost would be the development of standardized inoculation technique using immobilized spores. By using immobilized spores experiments could be designed to determine the effect of the type of growth medium used and the age of the inocula on subsequent shake flask and fermenter cultures. The size of the immobilized beads could also be varied thereby optimizing the size and weight of the inoculum. Also studies on biomass accumulation could be repeated to determine the correlation between dry weight and pellet size and settled volume.

Further analysis of fed batch cultures should determine what is limiting CPO production levels. If a feed can be given at the right time to stimulate another round of CPO mRNA synthesis and transcription, the final CPO yield may be elevated. Experiments still need to be carried out to fine tune the continuous feed solution to minimize 'waste' carbohydrate and growth yet stabilize the CPO synthesis machinery.

A possibe procedure for controlling enzyme production would involve the use a fructose feed to a fermenter culture growing on the defined salts medium. Growth of the fungus would then be determined by the flowrate of the feed solution and the concentration of the fructose. Alternatively, a nitrate feed to a culture growing on FS (minus nitrate) may give a different form of control.

Additional bioreactor redesign for temperature, foaming, pH and pO_2 monitoring and control would aid in the experimenter's control of the fermentation. This environmentally directed control may allow for improvements in the qualitative and quantitative aspects of the fermentation.

Another study of interest would involve microscopic morphology analysis. Light and electron microscope studies on the morphology of good and poor CPO producing strains of *C. fumago* and *Leptoxyphium* sp. may detect ultrastructural differences that could account for variations in CPO production.

5. REFERENCES

Aibara, S., Yamashita, H., Mori, E., Kato, M., and Morita, Y. 1982. Isolation and characterization of five neutral isoenzymes of horseradish peroxidase. J. Biochem. **92**: 531-539.

AiresBarros, M.R., Cabral, J.M.S., and Novais, J.M. 1987. Production of ethanol by immobilized *Saccharomyces bayanus* in an extractive fermentation system. Biotechnol. Bioeng. **29**: 1097-1104.

Alberti, B.N., and Klibanov, A.M. 1981. The enzymatic removal of dissolved aromatics from industrial aqueous effluents. Biotechnol. Bioeng. Symp. **11:** 373-379.

Anderson, J.G. 1983. Immobilized cell and film reactor systems for filamentous fungi. In The Filamentous Fungi Vol. IV Fungal Technology, Smith, J.E., D.R. Berry and B. Kristiansen (eds.), Edward Arnold (Publishers) Ltd., London, pp.145-170.

Anselmo, A.M., Mateus, M., Cabral, J.M.S., and Novais, J.M. 1985. Degradation of phenol by immobilized cells of *Fusarium flocciferum*. Biotechnol. Lett. **7**: 889-894.

Ashley, P.L., and Griffin, B.W. 1981. Chloroperoxidase-catalyzed halogenation of antipyrine, a drug substrate of liver microsomal cytochrome P-450. Arch. Biochem. Biophys. **210**: 167-178.

Atlow, S.C., Bonadonna-Aparo, L., and Klibanov, A.M. 1984. Dephenolization of industrial wastewaters catalyzed by polyphenol oxidase. Biotechnol. Bioeng. **26**: 599-603.

Axley, M.J., Kenigsberg, P., and Hager, L.P. 1986. Fructose induces and glucose represses chloroperoxidase mRNA levels. J. Biol. Chem. **261**: 15058-15061.

Babich, H., and Davis, D.L. 1981. Phenol: A review of environmental and health risks. Regul. Toxicol. Pharmacol. 1: 90-109.

Beckwith, J.R., and Hager, L.P. 1963. Biological chlorination VIII. Late intermediates in the biosynthesis of caldariomycin. J. Biol. Chem. **238**: 3091-3094.

Bisping, B., and Rehm, H.J. 1982. Glycerol production by immobilized cells of *Saccharomyces cerevisiae*. Eur. J. Appl. Microbiol. Technol. **14**: 136-139.

Bollag, J.M., and Liu, S.Y. 1985. Copolymerization of halogenated phenols and syringic acid. Pestic. Biochem. Physiol. **23**: 261-272.

Booth, H., and Saunders, B.C. 1956. Studies in peroxidase action. Part X. The oxidation of phenols. J. Chem. Soc. (London) Part 1, pp. 940-948.

Bucke, C. 1983. Practicality of industrial enzymes. Biochem. Soc. Trans. **11:** 13-14.

Burkholder, P.R., and Sinnott, E.W. 1945. Morphogenesis of fungal colonies in submerged shaken cultures. Am. J. Bot. **32**: 424-431.

Carmichael, J.W., Kendrick, W.B., Conners, I.L., and Sigler, L. 1980. Genera of Hyphomycetes, University of Alberta Press, Edmonton, Alberta. p. 118 and 262.

Carmichael, R.D., Jones, A., and Pickard, M.A. 1986. Semicontinuous and continuous production of chloroperoxidase by *Caldariomyces fumago* immobilized in κ-carrageenan. Appl. Environ. Microbiol. **51**: 276-280.

Chance, B. 1952. Effect of pH upon the reaction kinetics of the enzyme-substrate compounds of catalase. J. Biol. Chem. **194**: 471-482.

Chapman, P.J. 1972. An outline of reaction sequences used for the bacterial degradation of phenolic coumpounds. In Degradation of Synthetic Organic Molecules in the Biosphere. NAS, Washington, D.C.

Chapsal, J.M., Bourbigot, M.M., and Thomas, D. 1986. Oxidation of aromatic compounds by haemoglobin. Water. Res. **20**: 709-713.

Cheetham, P.S.J., and Tate and Lyle Ltd. 1980. Developments in the immobilization of microbial cells and their applications. In Topics in Enzyme and Fermentation Biotechnology, A. Wiseman (ed.), John Wiley and Sons, New York, pp.189-238.

Chibata, I., and Tosa, T. 1981. Use of immobilized cells. Annu. Rev. Biophys. Bioeng. **10**: 197-216.

Chibata, I., Tosa, T., and Sato, T. 1983. Immobilized cells in preparation of fine chemicals. Adv. Biotechnol. Processes **1**: 203-222.

Chien, N.K., and Sofer, S.S. 1985. Flow rate and bead size as critical parameters for immobilized-yeast reactors. Enzyme Microb. Technol. **7**: 538-542.

Clark, S. 1962. Submerged citric acid fermentation of ferrocyanide-treated beet molasses: morphology of pellets of *Aspergillus niger*. Can. J. Microbiol. **8**: 133-136.

Clutterbuck, P.W., Mukhopadhya, S.L., Oxford, A.E., and Raistrick, H. 1940. Studies in the biochemistry of micro-organisms. 65(A) A survey of chlorine metabolism by moulds. (B) Caldariomycin, $C_5H_8O_2Cl_2$, a metabolic product of *Caldariomyces fumago* Woronichin. Biochem. J. **34**: 664-677.

Corbett, M.D., and Chipko, B.R. 1979. Peroxide oxidation of indole to oxindole by chloroperoxidase catalysis. Biochem. J. **183**: 269-276.

Corbett, M.D., Chipko, B.R., and Batchelor, A.O. 1980. The action of chloride peroxide on 4-chloroaniline. Biochem. J. **187**: 893-903.

Cunningham, J.E., and Pickard, M.A. 1985. Maltol, a metabolite of *Scytalidium uredinicola* which inhibits spore germination of *Endocronartium harknessii*, the western gall rust. Can. J. Microbiol. 31: 1051-1055.

Das, R.C., and Shultz, J.L. 1987. Secretion of heterologous proteins from *Saccharomyces cerevisiae*. Biotechnol. Prog. 3: 43-48.

Dawson, J.H., Kau, L.-S., Penner-Hahn, J.E., Sono, M., Eble, K.S., Bruce, G.S., Hager, L.P., and Hodgson, K.O. 1986. Oxygenated cytochrome P-450-cam and chloroperoxidase: Direct evidence for sulfur donor ligation trans to dioxygen and structural characterization using EXAFS spectroscopy. J. Am. Chem. Soc. **108**: 8114-8116.

Demain, A.L., and Solomon, N.A. 1981. Industrial microbiology. Scientific American **245**: 67-75.

Deo, Y.M., Costerton, J.W., and Gaucher, G.M. 1983. Examination of immobilized fungal cells by phase-contrast and scanning electron microscopy. Can. J. Microbiol. **29**:1642-1649.

Deo, Y.M., and Gaucher, G.M. 1983. Semi-continuous production of the antibiotic patulin by immobilized cells of *Penicillium urticae*. Biotechnol. Lett. **5**: 125-130.

Deo, Y.M., and Gaucher, G.M. 1985. Effect of nitrogen supplementation on the longevity of antibiotic production of immobilized cells of *Penicillium urticae*. Biotechnol. Lett. **21**: 220-227.

Deshpande, M.V., Balkrishnan, H., Ranjekar, P.K., and Shankar, V. 1987. Isolation and immobilization of *Sclerotium rolfsii* protoplasts. Biotechnol. Lett. 9: 49-52.

Dubois, M., Gilles, K.A., Hamilton, I.K., Rebers, P.A., and Smith, F. 1956. Colorimetric method for the determination of sugars and related substances. Anal. Chem. **28**: 350-355.

Dumontet, C., and Rousset, B. 1983. Identification, purification, and characterization of a non-heme lactoperoxidase in bovine milk. J. Biol. Chem. **258**: 14166-14172.

Dunford, H.B., Lambeir, A.M., Kashem, M.A., and Pickard, M.A. 1987. On the mechanism of chlorination by chloroperoxidase. Arch. Biochem. Biophys. **252**: 292-302.

Edeline, F., Lambert, G., and Fatticcioni, H. 1986. Anaerobic treatment of coke plant waste water. Process. Biochem. **21**: 58-60.

Falb, R.D. 1972. Immobilization of enzymes: An overview. Biotechnol. Bioeng. Symp. **3**: 177-184 , In Enzyme Engineering, L.B. Wingard (ed), John Wiley and Sons, Inc., New York.

Fang, G.H., Kenigsberg, P., Axley, M.J., Nuell, M., and Hager, L.P. 1986. Cloning and sequencing of chloroperoxidase cDNA. Nucl. Acids Res. **14**: 8061-8071.

Fedorak, P.M. 1984. Anaerobic biological treatment of phenolic wastewaters. Ph.D. Thesis, University of Alberta.

Fedorak, P.M., and Hrudey, S.E. 1984a. The effects of phenol and some alkyl phenolics on batch anaerobic methanogenesis. Water Res. **18**: 361-367.

Fedorak, P.M., and Hrudey, S.E. 1984b. Batch anaerobic methanogenesis of phenolic coal conversion wastewater. Water Sci. Technol. **17**: 143-154.

Fedorak, P.M., and Hrudey, S.E. 1986. Anaerobic treatment of phenolic coal conversion wastewater in semicontinuous cultures. Water Res. **20**: 113-122.

Fedorak, P.M., and Hrudey, S.E. 1987. Inhibition of anaerobic degradation of phenolics and methanogenesis by coal coking wastewater. Water Sci. Technol. **19**: 219-228.

Frein, E.M., Montenecourt, B.S., and Eveleigh, D.E. 1982. Cellulase production by *Trichoderma reesei* immobilized on *k* -carrageenan. Biotechnol. Lett. **4**: 287-292.

Gaden, Jr., E.L. 1981. Production methods in industrial microbiology. Scientific American **245**: 181-196.

Gaucher, G.M., Lam, K.S., Grootwassink, J.W.D., Neway, J., and Deo, Y.M. 1981. The initiation and longevity of patulin biosynthesis. In Developments in Industrial Microbiology, vol. **22**, Soc. Ind. Microbiol, pp. 219-232.

Geigert, J., Neidleman, S.L., Demetrios, D.J., and DeWitt, S.K. 1983a. Haloperoxidases: Enzymatic synthesis of α,β-halohydrins from gaseous alkenes. Appl. Environ. Microbiol. **45**: 366-374.

Geigert, J., Demetrios, D.J., Neidleman, S.L., Lee, T D., and Wadsworth, J. 1983b. Peroxide oxidation of primary alcohols to aldehydes by chloroperoxide catalysis. Biochem. Biophys. Res. Commun. **114**: 1104-1108.

Geigert, J., DeWitt, S.K., Neidleman, S.L., Lee, G., Demetrios, D.J., and Moreland, M. 1983c. DMSO is a substrate for chloroperoxidase. Biochem. Biophys. Res. Commun. **116**: 82-85.

Geigert J., Hunter, J.C., and Neidleman, S.L. 1983d. The discovery of a new ecological niche for halogenating enzymes. In Proceedings of the International Conference on Enzyme Engineering VII, abstract, p. 27.

Geigert J., and Neidleman, S.L. 1985. Enzymatic synthesis of halohydrins and their conversion to epoxides. In Enzymes and Immobilized Cells in Biotechnology, A.I. Laskin (ed.), The Benjamin/Cummings Publishing Co., Inc., London, pp. 283-296.

Geigert, J., and Neidleman, S.L. 1986. Vicinal heterogeneous dihalogenated products and method. U.S. Patent Office, Patent #4587217, 435, 155.

Geigert, J., Lee, T.D., Dalietos, D.J., Hirano, D.S., and Neidleman, S.L. 1986. Epoxidation of alkenes by chloroperoxidase catalysis. Biochem. Biophys. Res. Commun. **136**: 778-782.

Godia, F., Casas, C., and Sola, C. 1987. A survey of continuous ethanol fermentation systems using immobilized cells. Process Biochem. **22**: 43-48.

Gonzales-Vergara, E., Ales, D.C., and Goff, H.M. 1986. A simple, rapid, high-yield isolation and purification procedure for chloroperoxidase isoenzymes. Prep. Biochem. **15**: 335-348.

Gray, R.P., and Vu-Trong, K. 1987. Production of the macrolide antibiotic tylosin in cyclic fed-batch culture. Biotechnol. Bioeng. **29**: 33-40.

Griffin, B.W. 1983. Mechanism of halide-stimulated activity of chloroperoxidase. Evidence for enzymatic formation of free hypohalous acid. Biochem. Biophys. Res. Commun. **116**: 873-879.

Griffin, B.W., and Ashley, P.L. 1984. Evidence for a radical mechanism of halogenation of monochlorodimedone catalyzed by chloroperoxidase. Arch. Biochem. Biophys. **233**: 188-196.

Hager, L.P., Morris, D.R., Brown, F.S., and Eberwein, H. 1966. Chloroperoxidase II. Utilization of halogen anions. J. Biol. Chem. **241**: 1769-1777.

Hashimoto, A., and Pickard, M.A. 1984. Chloroperoxidases from *Caldariomyces (=Leptoxyphium)* cultures: glycoproteins with variable carbohydrate content and isoenzymic forms. J. Gen. Microbiol. **130:** 2051-2058.

Heinrich, M., and Rehm, H.J. 1982. Formation of gluconic acid at low pH-values by free and immobilized *Aspergillus niger* cells during citric acid fermentation. Eur. J. Appl. Microbiol. Biotechnol. **15**: 88-92.

Ho, C.S., Baddour, R.F., and Wang, D.I.C. 1984. Effective diffusivity of oxygen in microbial pellets. Biotechnol. Adv. **2**: 21-33.

Hewson, W.D., and Hager, L.P. 1980. Bromoperoxidases and halogenated lipids in marine algae. J. Phycol. **16**: 340-345.

Hollenberg, P.F., and Hager, L.P. 1973. The P-450 nature of the carbon monoxide complex of ferrous chloroperoxidase. J. Biol. Chem. **248**: 2630-2633.

Hollenberg, P.F., and Hager, L.P. 1978. Purification of chloroperoxidase from *Caldariomyces fumago*. Methods in Enzymology **52**: 521-529.

Horitsu, H., Takahashi, Y., Tsuda, J., Kawai, K., and Kawano, Y. 1983. Production of itaconic acid by *Aspergillus terreus* immobilized in polyacrylamide gels. Eur. J. Appl. Microbiol. Biotechnol. **18**: 358-360.

Hughes, S.J. 1976. Sooty Moulds. Mycologia **68**: 693-820.

Itoh, N., Cheng, L.Y., Izumi, Y., and Yamada, H. 1987. Immobilized bromoperoxidase of *Corallina pilulifera* as a multifunctional halogenating biocatalyst. J. Biotechnol. **5**: 29-38.

Jager, A., Croan, S., and Kirk, T.K. 1985. Production of ligninases and degradation of lignin in agitated submerged cultures of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **50**: 1274-1278.

Jensen, D.E. 1972. Continuous production of extracellular protease by *Bacillus subtilis* in a two-stage fermentor. Biotechnol. Bioeng. **14**: 647-662.

Jensen, S.E., Westlake, D.W.S., and Wolfe, S. 1984. Production of penicillins and cephalosporins in an immobilized enzyme reactor. Appl. Microbiol. Biotechnol. **20**: 155-160.

Jones, A., Berk, D., Lesser, B.H., Behie, L.A., and Gaucher, G.M. 1983. Continuous production of patulin by immobilized cells of *Penicillium urticae* in a stirred tank reactor. Biotechnol. Lett. **5**: 785-790.

Joshi, S., and Yamazaki, H. 1987. Resistence of bacterial film to contamination during use as inoculum in repeated batch fermentation. Biotechnol. Lett. **9**: 43-46.

Ju, N., and Wang, S.S. 1986. Continuous production of itaconic acid by Aspergillus terreus in a porous disk bioreactor. Appl. Microbiol. Biotechnol. **23**: 311-314.

Kahlon, S.S., and Malhotra, S. 1986. Production of gibberellic acid by fungal mycelium immobilized in sodium alginate. Enzyme Microb. Technol. **8**: 613-616.

Kanofsky, J.R. 1984. Singlet oxygen production by chloroperoxidase-hydrogen peroxide-halide systems. J. Biol. Chem. **259**: 5596-5600.

Kawakami, K., Nagamatsu, S., Ishii, M., and Kusunoki, K. 1986. Enzymatic formation of propylene bromohydrin from propylene by glucose oxidase and lactoperoxidase. Biotechnol. Bioeng. **28**: 1007-1013.

Kenigsberg, P., Fang, G.-H., and Hager, L.P. 1987. Post-translational modifications of chloroperoxidase from *Caldariomyces fumago*. Arch. Biochem. Biophys. **254**: 409-415.

Khan, K.A., Suidan, M.T., and Cross, W.H. 1981. Anaerobic activated carbon filter for the treatment of phenol-bearing wastewater. J. Water Pollut. Control Fed. **53**: 1519-1532.

Kierstan, M., and Bucke, C. 1977. The immobilization of microbial cells, subcellular organelles and enzymes in calcium alginate gels. Biotechnol. Bioeng. **19**: 387-397.

Kirk, T.K., Croan, S., Tien, M., Murtagh, K.E., and Farrell, R.L. 1986. Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. 8: 27-32.

Klibanov, A.M. 1983. Immobilized enzymes and cells as practical catalysts. Science **219**: 722-727.

Klibanov, A.M., and Morris, E.D. 1981. Horseradish peroxidase for the removal of carcinogenic aromatic amines from water. Enzyme Microb. Technol. **3**: 119-122.

Klibanov, A.M., Tu, T.M., and Scott, K.P. 1983. Peroxidase-catalyzed removal of phenols from coal-conversion waste waters. Science **221**: 259-261.

Kloosterman, J., and Lilly, M.D. 1985. An airlift loop reactor for the transformation of steroids by immobilized cells. Biotechnol. Lett. **7**: 25-30.

Kokubu, T., Karube, I., and Suzuki, S. 1978. Alpha -amylase production by immobilized whole cells of *Bacillus subtilis*. Eur. J. Appl. Microbiol. Biotechnol. **5**: 233-240.

Kokubu, T., Karube, I., and Suzuki, S. 1981. Protease production by immobilized mycelia of *Streptomyces fradiae*. Biotechnol. Bioeng. **23**: 29-39.

Konig, B., Schugerl, K., and Seewald, C. 1982. Stategies for penicillin fermentation in tower loop reactors. Biotechnol. Bioeng. **24**: 259-280.

Krejcarek, G.E., Bryant, R.G., Smith, R.J., and Hager, L.P. 1976. Broad-line nuclear magnetic resonance studies of chloroperoxidase. Biochemistry **15**: 2508-2511.

Laane, C., Weyland, A., and Franssen, M. 1986. Bioelectrosynthesis of halogenated compounds using chloroperoxidase. Enzyme Microb. Technol. **8**: 345-348.

Lambeir, A.M., and Dunford, H.B. 1983. A steady state kinetic analysis of the reaction of chloroperoxidase with peracetic acid, chloride and 2-chlorodimedone. J. Biol.

Chem. **258**: 13558-13563.

Lambeir, A.M., Dunford, H.B., and Pickard, M.A. 1983. Kinetics of cyanide binding to chloroperoxidase in the presence of nitrate: Detection of the influence of a heme-linked acid group by shift in the apparent pKa value. J. Inorgan. Biochem. **19**: 291-300.

Lambert, P.W. 1983. Industrial enzyme production and recovery from filamentous fungi. In The Filamentous Fungi Vol. IV Fungal Technology, Smith, J.E., D.R. Berry and B. Kristiansen (eds.), Edward Arnold (Publishers) Ltd., London, pp. 210-237.

Lee, V. 1984. Creating new enzymes with chemistry. Bio-Technology **2**: 549-551.

Libby, R.D., Thomas, J. A., Kaiser, L.W., and Hager, L.P. 1982. Chloroperoxidase halogenation reactions. Chemical versus enzymic halogenating intermediates. J. Biol. Chem. **257**: 5030-5037.

Linko, Y.Y., Jalanka, H., and Linko, P. 1981. Ethanol production from whey with immobilized living yeast. Biotechnol. Lett. **3**: 263-268.

Linko, P., and Linko, Y.Y. 1984. Industrial applications of immobilized cells. CRC Crit. Rev. Biotechnol. **1**: 289-338.

Linko, Y.Y., Leisola, M., Lindholm, N., Troller, J., Linko, P., and Fiechter, A. 1986. Continuous production of lignin peroxidase by *Phanerochaete chrysosporium*. J. Biotechnol. **4**: 283-291.

Liu, T.N.E., Mtimkulu, T., Geigert, J., Wolf, B., Neidleman, S.L., Silva, D., and Hunterce, J.C. 1987. Isolation and characterization of a novel non-heme chloroperoxidase. Biochem. Biophys. Res. Commun. **142**: 329-333.

Livernoche, D., Jurasek, L., Desrochers, M., and Dorica, J. 1983. Removal of color from kraft mill wastewaters with cultures of white-rot fungi and with immobilized mycelium of *Coriolus versicolor*. Biotechnol. Bioeng. **25**: 2055-2065.

Maloney, S.W., Manem, J., Mallevialle, J., and Flessinger, F. 1984. The potential use of enzymes for the removal of aromatic compounds from water. Water Sci. Technol. **17**: 273-278.

Maloney, S.W., Manem, J., Mallevialle, J., and Flessinger, F. 1986. Transformation of trace organic compounds in drinking water by enzymatic oxidative coupling. Environ. Sci. Technol. **20**: 249-253.

Manner, M.J. 1977. Advanced Wastewater Treatment. In Water and Wastewater Technology, John Wiley and Sons, New York, pp. 450-460.

Marek, M., Valentova, O., and Kas, J. 1984. Invertase immobilization via its carbohydrate moiety. Biotechnol. Bioeng. **26**: 1223-1226.

Martin, A.M., and Bailey, V.I. 1985. Growth of *Agaricus campestris* NRRL 2334 in the form of pellets. Appl. Environ. Microbiol. **49**: 1502-1506.

Martin, M., and Sabater, B. 1981. Cell-free synthesis of catalytically active fructokinase directed by RNA from *Streptomyces violaceoruber* grown with fructose. J. Gen. Microbiol. **126**: 203-210.

Martin, S.M., and Waters, W.R. 1952. Production of citric acid by submerged fermentation. Ind. Eng. Chem. **39**: 2229-2233.

Martyn, R.P., Branzei, S.C., and Sperl, G.T. 1981. Antimicrobial chlorinated β-diketones. Bios **52**: 8-12.

Meers, J.L. 1981. The continuous production of glucose isomerase. Biotechnol. Lett. **3**: 131-136.

Messing, R.A. (Ed). 1975. Immobilized Enzymes for Industrial Reactors. Academic

Press, New York.

Messing, R.A. 1982. Immobilized microbes and a high rate, continuous waste processor for the production of high Btu gas and the reduction of pollutants. Biotechnol. Bioeng. **24:** 1115-1123.

Metcalf and Eddy, Inc. 1979. Wastewater engineering: treatment, disposal, reuse. Second Ed. McGraw-Hill, Inc., pp. 156-160.

Metz, B., and Kossen, N.W. 1977. The growth of molds in the form of pellets. A literature review. Biotechnol. Bioeng. **19**: 781-799.

Mitchell, E.D. Jr., Loring, R., Carraway, K.L., and Cunningham, B. 1981. Oxidative inactivation of *B. subtilis* α-amylase by chloroperoxidase. Proc. Okla. Acad. Sci. **61**: 43-48.

Morris, D.R., and Hager, L.P. 1966. Chloroperoxidase I. Isolation and properties of the crystalline glycoprotein. J. Biol. Chem. **241:** 1763-1768.

Morrison, M., and Schonbaum, G.R. 1976. Peroxidase-catalyzed halogenation. Annu. Rev. Biochem. **45**: 861-888.

Murata, K., Tani, K., Kato, J., and Chibata, I. 1981. Glutathione production by immobilized *Saccharomyces cerevisiae* cells containing an ATP regeneration system. Eur. J. Appl. Microbiol. Biotechnol. **11**: 72-77.

Neidleman, S.L. 1975. Microbial halogenation. CRC Crit. Rev. Microbiol. **5**: 333-358.

Neidleman, S.L. 1984. Waste-water cleanup with horseradish peroxidase. Trends in Biotechnol. **2**: 107-108.

Neidleman, S.L., and Geigert, J. *1981a.* Method for producing iodine. U.S. Patent Office, Patent #4, 282, 324.

Neidleman, S.L., Amon Jr., W.F., and Geigert, J. 1981b. Preparation of epoxides and glycols from gaseous alkenes. U.S. Patent Office, Patent #4, 284, 723.

Neidleman, S.L., and Geigert, J. 1983. Biological halogenation and epoxidation. Biochem. Soc. Symp. 48: 39-52 . In Biotechnology, C.F. Phelps and P.H. Clarke (ed.) The Biochemical Society, London.

Neidleman, S.L., and Geigert, J. 1986. Biohalogenation: Principles, Basic Roles and Applications. Ellis Horwood Limited, John Wiley and Sons, New York, pp 1-200.

Neufeld, R.D. 1984. The treament of wastes from the synthetic fuels industry. In Surveys in Industrial Wastewater Treatment Vol. 2, D. Barnes, C. Forster, and S.E. Hrudey (eds.), Pitmans, London, pp. 65-129.

Neway, J., and Gaucher, G.M. 1981. Intrinsic limitations on the continued production of the antibiotic patulin by *Penicillium urticae* . Can. J. Microbiol. **27**: 206-215.

Orndorff, S.A. 1983. Enzymatic catalyzed biocide system. U.S. Patent Office, Patent #4, 370, 199.

Paice, M.G., and Jurasek, L. 1984. Peroxidase-catalyzed color removal from bleach plant effluent. Biotechnol. Bioeng. **26**: 477-480.

Petty, M.A. 1961. An introduction to the origin and biochemistry of microbial halometabolites. Bacteriol. Rev. **25**: 111-130.

Phaff, H.J. 1981. Industrial Microorganisms. Scientific American **245**: 76-89.

Pickard, M.A. 1981. A defined growth medium for the production of chloroperoxidase by *Caldariomyces fumago*. Can. J. Microbiol. **27**:1298-1305.

Pickard, M.A., and Hashimoto, A. 1982. Isoenzymes of chloroperoxidase from *Caldariomyces fumago*. Can. J. Microbiol. **28**: 1382-1388.

Pirt, S.J. 1975. Principles of microbe and cell cultivation. Blackwell Scientific Publications, Halsted Press, John Wiley and Sons, New York, pp. 156-169.

Priest, F.G. 1983. Enzyme synthesis: Regulation and process of secretion by microorganisms. In Microbial Enzymes and Biotechnology, W.M. Fogarty (ed.), Applied Science Publishers, London, pp. 319-366.

Ramachandran, S., and Gottlieb, D. 1963. Pathways of glucose catabolism in *Caldariomyces fumago*. Biochim. Biophys. Acta **69**: 74-84.

Ramakrishnan, K., Oppenhuizen, M.E., Saunders, S., and Fisher, J. 1983. Stereo-selectivity of chloroperoxidase-dependent halogenation. Biochem. **22**: 3271-3277.

Remba, R.D., Champion, P.M., Fitchen, D.B., Chang, R., and Hager, L.P. 1979. Resonance Raman investigations of chloroperoxidase, horseradish peroxidase, and cytochrome c using soret band laser excitation. Biochem. **18**: 2280-2290.

Russell, T.W.F., and Dunn, I.J. 1974. The tubular loop batch fermenter: Basic concepts. Biotechnol. Bioeng. **16**:1261-1272.

Sae, A. S. W., and Cunningham, B. A. 1979. Isolation and properties of chloroperoxidase isoenzymes. Phytochemistry **18**:1785-1787.

Sato, T., Nishida, Y., Tosa, T., and Chibata, I. 1979. Immobilization of *Escherichia coli* cells containing aspartase activity with κ-carrageenan. Enzymic properties and appliction for L-aspartic acid production. Biochim. Biophys. Acta **570**: 179-186.

Schugerl, K., Wittler, R., and Lorenz, T. 1983. The use of molds in pellet form. Trends in Biotechnol. **1**: 120-122.

Scott, C.D. 1987. Immobilized cells: a review of recent literature. Enzyme Microb. Technol. **9**: 66-73.

Shahangian, S., and Hager, L.P. 1981. The reaction of chloroperoxidase with chlorite and chlorine dioxide. J. Biol. Chem. **256**: 6034-6040.

Shaw, P.D., and Hager, L.P. 1959a. An enzymatic chlorination reaction. J. Am. Chem. Soc. **80**: 1011-1012.

Shaw, P.D., and Hager, L.P. 1959b. Biological chlorination. IV. Peroxidative nature of enzymatic chlorination. J. Am. Chem. Soc. **81**: 6527-6528.

Shaw, P.D., and Hager, L.P. 1961. Biological chlorination VI. Chloroperoxidase: a component of the β-ketoadipate chlorinase system. J. Biol. Chem. **236**: 1626-1630.

Shuttleworth, K.L., and Bollag, J.-M. 1986. Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. Enzyme Microb. Technol. **8**: 171-177.

Steel, R., Martin, S.M., and Lentz, C.P. 1954. A standard inoculum for citric acid production in submerged culture. Can. J. Microbiol. **1**: 150-157.

Suter, I.I., Vartek, H.G., Srinivasan, M.C., and SivaRaman, H. 1986. Production of alkaline protease by immobilized mycelium of *Conidiobolus*. Enzyme Microb. Technol. **8**: 632-634.

Thomas, J.A., and Hager, L.P. 1968. The peroxidation of molecular iodine to iodate by chloroperoxidase. Biochem. Biophys. Res. Commun. **32**: 770-775.

Thomas, J.A., Morris, D.R., and Hager, L.P. 1970a. Chloroperoxidase VII. Classical peroxidatic, catalatic, and halogenating forms of the enzyme. J. Biol. Chem. **245**: 3129-3134.

Thomas, J.A., Morris, D.R., and Hager, L.P. 1970b. Chloroperoxidase VIII. Formation of peroxide and halide complexes and their relation to the mechanism of the halogenation reaction. J. Biol. Chem. **245**: 3135-3142.

Tosa, T., Sato, T., Mori, T., Yamamoto, K., Takata, I., Nishida, Y., and Chibata, I. 1979. Immobilization of enzymes and microbial cells using carrageenan as matrix. Biotechnol. Bioeng. **21:** 1697-1709.

Tsay, S.S. and To, K.Y. 1987. Citric acid production using immobilized conidia of *Aspergillus niger* TMB 2022. Biotechnol. Bioeng. **29**: 297-304.

van den Heuvel, J.C., Zoetemeyer, R.J., and Boelhouwer, C. 1981. Purification of municipal wastewater by subsequent reverse osmosis and anaerobic digestion. Biotechnol. Bioeng. **23**: 2001-2008.

van Pee, K.H., and Lingens, F. 1985. Purification of bromoperoxidase from Pseudomonas aureofaciens. J. Bacteriol. **161**: 1171-1175.

van Suijdam, J.C., Kossen, N.W.F., and Paul, P.G. 1980. An inoculum technique for the production of pellets. Eur. J. Appl. Microbiol. Biotechnol. **10**: 211-221.

van Suijdam, J.C., and Metz, B. 1981. Influence of engineering variables upon the morphology of filamentous molds. Biotechnol. Bioeng. **23**: 111-148.

Vu-Trong, K., and Gray, P.P. 1982. Stimulation of tylosin productivty resulting from cyclic feeding profiles in fed batch culture. Biotechnol. Lett. **4**: 725-728.

Vu-Trong, K., and Gray, P.P. 1986. Patterns of RNA synthesis in batch and cyclic fed-batch cultures of tylosin-producing *Streptomyces fradiae*. Biotechnol. Lett. **8**: 849-852.

Ward, E.W.B., and Colotelo, N. 1960. The importance of inoculum standardization in nutritional experiments with fungi. Can. J. Microbiol. **6**: 545-556.

Wase, D.A., McManamey, W.J., Raymahasay, S., and Vaid, A.K. 1985. Comparisons between production by *Aspergillus fumigatus* in agitated vessels and in an air-lift fementor. Biotechnol. Bioeng. **27**: 1166-1172.

Webber, D. 1984. Top fifty chemical products. Chem. Engng. News **62**: 8-10.

Westerfeld, W.W., and Lowe, C. 1942. The oxidation of p-cresol by peroxidase. J. Biol. Chem. **145**: 463-470.

Westmeier, F., and Rehm, H.J. 1987. Degradation of 4-chlorophenol in municipal wastewater by adsorptive immobilized *Alcaligenes* sp. A7-2. Appl. Microbiol. Biotechnol. **26**: 78-83.

Wever, R., Plat, H., and de Boer, E. 1985. Isolation procedure and some properties of the bromoperoxidases from the seaweed *Ascophyllum nodosum*. Biochim. Biophys. Acta. **830**: 181-186.

Whitaker, A., and Long, P.A. 1973. Fungal Pelleting. Process Biochem. **8**: 27-31.

Wiesner, W., van Pee, K.H., and Lingens, F. 1986. Detection of a new chloroperoxidase in *Pseudomonas pyrrocinia*. FEBS Lett. **209**: 321-324.

Wingard, L.B. Jr. (Ed). 1972. Enzyme Engineering. John Wiley and Sons, New York.

Witholt, B., Lageveen, R.G., and Kok, M. 1985. Future applications including protein engineering. In Biocataysts in Organic Syntheses, J. Tramper, H.C. van der Plas, and P. Linko (eds.), Elsevier Science Publishers, New York, pp. 239-258.

Wolfe, S., Demain, A.L., Jensen, S.E., and Westlake, D.W.S. 1984. Enzymatic approach to syntheses of unnatural beta-lactams. Science **226**: 1386-1392.

Wood, L.L., and Calton, G.J. 1984. A novel method of immobilization and its use in aspartic acid production. Biotechnology **2**: 1081-1084.

Woronichin, N.N. 1926. Zur kenntnis der Morphologie und Systematik der

Russtaupitze Transkaukasiens. Annales mycologici (Berlin) **24**: 231-264.

Yamada, H., Itoh, N., Murakami, S., and Izumi, Y. 1985a. New bromoperoxidases from coralline algae that brominates phenol compounds. Agric. Biol. Chem. **49**: 2961-2967.

Yamada, H., Itoh, N., and Izumi, Y. 1985b. Chloroperoxidase-catalyzed halogenation of trans-cinnamic acid and its derivatives. J. Biol. Chem. **260**: 11962-11969.

Yoshizumi, K., Aoki, K., Matsuoka, T., and Asakura, S. 1985. Determination of nitrate by a flow system with a chemiluminescent NO_x analyzer. Anal. Chem. **57**: 737-740.

Younes, G., Breton, A.M., and Guespin-Michel, J. 1987. Production of extracellular native and foreign proteins by immobilized growing cells of *Myxococcus xanthus*. Appl. Microbiol. Biotechnol. **26**: 507-512.

Zahner, H., and Kurth, R. 1982. Overproduction of microbial metabolites-The supply of precursors from the intermediary metabolism. In Overproduction of Microbial Metabolites, V. Krumphanzl, B. Sikyta, and Z. Vanek (eds.), Academic Press (London), pp. 167-179.